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SULFAPYRIDINE: STUDIES ON ABSORPTION AND EXCRETION*

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SINCE the first of January, 1939, we have been using sulfapyridine† in the Louisville City and the Children's Free Hospitals. When we began to use it, little was known of its mode of action and its fate within the human body, the best method of its administration, its toxic effects, etc. We decided, therefore, to investigate such of these things as we could. Consequently, we have done blood and urine studies on a number of patients large enough to give us some information concerning those points. This report deals with the results of those studies.

MATERIAL

Sulfapyridine has been given to date to 131 patients, of whom 103 had pneumonia. On most of them, at least a few blood studies of sulfapyridine concentration have been made. Of this total number, 46 were studied thoroughly from the point of view of the concentration of the drug in the blood, its absorption and its elimination. Of the 46 cases, 38 had pneumonia and 8 had other conditions (1 empyema, 1 gonococcal arthritis, 1 typhoid fever, and 5 *Streptococcus viridans* endocarditis).

METHODS

The free and total sulfapyridine in blood and urine were determined by the method of Marshall. In the earlier studies the total sulfapyridine determinations were obviously in error, and are not used in this material. Later, when

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we came across Marshall's demonstration[1] that accuracy could be ensured by the use of ammonium sulfamate, especially when toluenesulfonic acid was used to precipitate the blood proteins, this method was followed and has been used ever since with results which have never given us occasion to suspect any inaccuracy. Throughout, we have had no reason to question the free sulfapyridine figures. Colorimetry was done with the Evelyn photoelectric colorimeter.

PROCEDURE

In 14 of the 46 cases the initial dose was 2 Gm. followed after four hours by 1 Gm. every four hours day and night. In the remaining 32 cases the initial dose was 4 Gm.; in 18 of these the second dose of 1 Gm. was given four hours later, and was then repeated every four hours day and night; in 14 cases the second dose was not given until eight hours later, after which it was continued every four hours as previously.

In 38 of the 46 cases blood was drawn for analysis one, two, three, and four hours after the initial dose. Thereafter, blood samples were taken at variable periods, but frequently at six, eight, twelve, and from twelve to twenty hours later. In the other 8 cases the first four hourly samples were omitted, but samples were taken every morning at 8 o'clock, so that in nearly every instance we have determinations made from thirty-two to forty-four hours after the initial dose. Since without exception the drug was given at 12, 4, 8, and 12 o'clock, on the hour, the 8 A.M. samples always represented the blood level four hours after the latest preceding dose.

Whenever possible to collect, 24-hour specimens of urine were sent to the laboratory every morning throughout the course of treatment. Since for one reason or another it was frequently impossible to collect all the urine every day in every case there were only 16 cases in which complete urine studies were possible. Collection of urine was continued until the drug had practically disappeared from it.

RESULTS

(a) *Absorption.* As all other workers who have studied this phase of the subject have observed, absorption of sulfapyridine is very irregular and unpredictable. Fig. 1 shows the absorption curves in the first 37 consecutive cases of this study. It can be seen at a glance that there is no uniformity in the shape or the height of the absorption curves from identical doses (e.g., Cases 9, 10, 11, and Cases 20, 21, 22, 23, and 33), nor is there any consistency of relationship in the absorption from 2 and from 4 Gm. (e.g., Cases 5 and 13, Cases 11 and 12, and Cases 31 and 32). It should be noted that neither Case 20 nor Case 29 had previously had either sulfapyridine or sulfanilamide, for this point was carefully investigated.

Fig. 2 shows the "scattering" of the individual values at one, two, three, and four hours after the administration of single doses of 2 and of 4 Gm. The lines connect the median points, giving what may be called typical curves. It will be noted that the curve following the 2 Gm. dose is even slightly higher than that following the 4 Gm. dose. It may be pointed out further, that if the

two highest readings for each period of the 4 Gm. dose were omitted, the general appearance of the two halves of the figure would be almost identical. Fig. 3 shows the typical curves (constructed from the median value for each period) carried on for a period of from thirty-three to forty-four hours after the initial dose. Here, too, the blood concentration of free sulfapyridine is higher following an initial dose of 2 Gm. than it is from 4 Gm., the maintenance dose being the same in both instances.

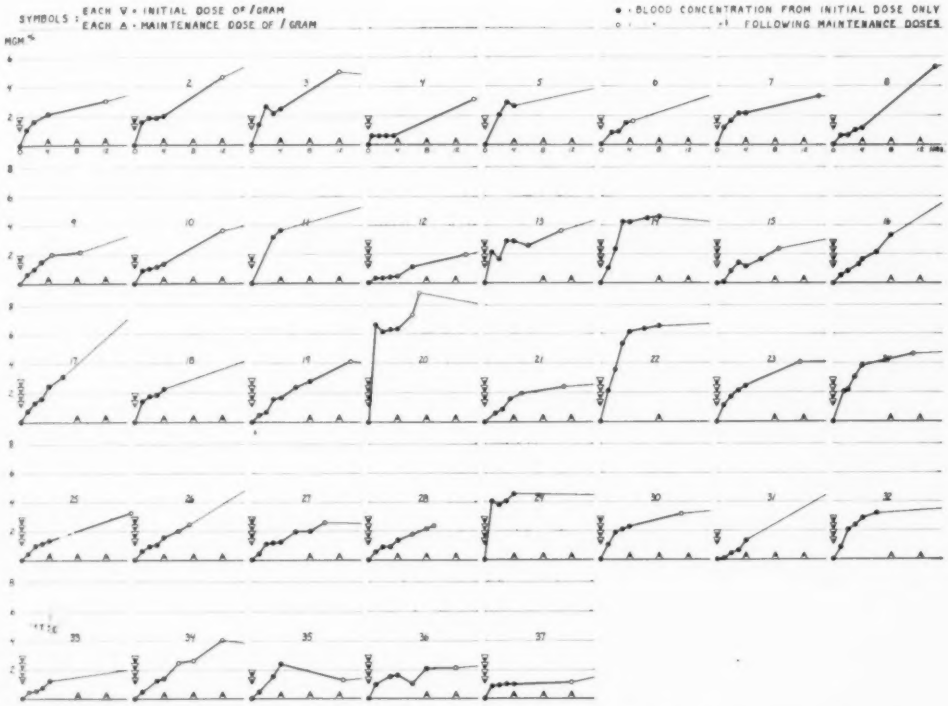
SYMBOLS: EACH ▽ = INITIAL DOSE OF 1 GRAM
EACH △ = MAINTENANCE DOSE OF 1 GRAM
● = BLOOD CONCENTRATION FROM INITIAL DOSE ONLY
○ = FOLLOWING MAINTENANCE DOSES
MGM%

Fig. 1.—The absorption curves of free sulfapyridine in the first 37 consecutive cases. Each arrowhead represents an oral dose of 1 Gm., the downwardly directed ones indicating the initial dose and the upwardly directed ones, maintenance doses. The solid dots indicate the blood concentration figures from the initial dose only, and the open circles from maintenance doses as well. The thin line at the end of each curve points to the next concentration point, some time later than fifteen hours after the initial dose.

This paradoxical situation is very interesting and of practical importance. A logical explanation would lie in the assumption that the larger dose produced vomiting, thus preventing further absorption of the drug. As a matter of fact, vomiting followed the larger doses (67 per cent of the cases) more frequently than it did the smaller ones (45 per cent of the cases). In the entire series 58.7 per cent vomited. However, a study of Fig. 4 will show that even where there was no vomiting, the curve is still much higher following the 2 Gm. than that following the 4 Gm. dose. The same thing is also true where there was vomiting. We, therefore, cannot explain the unexpected discrepancy between size of dose and height of the concentration curve on the factor of vomiting; it is possible that a very large number of cases would have reversed this trend, but in the group from which these figures were obtained there were 6 vomiting and 8 non-vomiting cases who received 2 Gm. as an initial dose, and 21 vomiting and 11

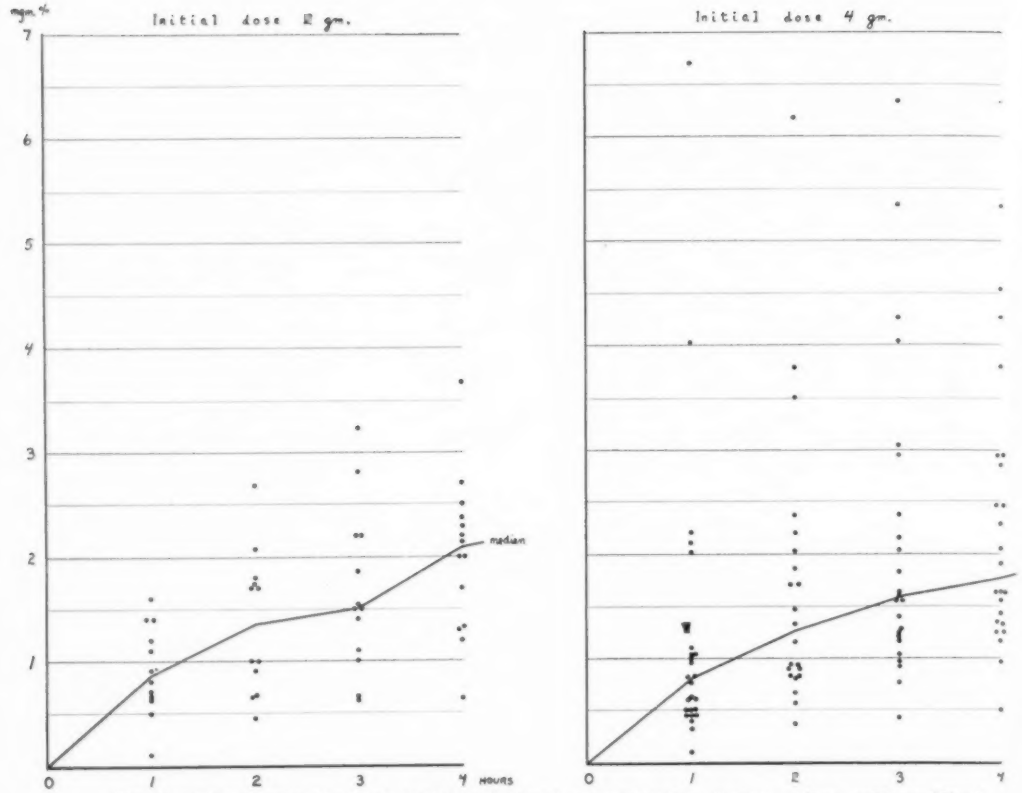

Fig. 2.—The "scattering" of individual blood concentration figures (free sulfapyridine) after single doses of 2 and 4 Gm. at one, two, three and four hours. Each point at each hour represents a different patient. The curve joins the median point of each group.

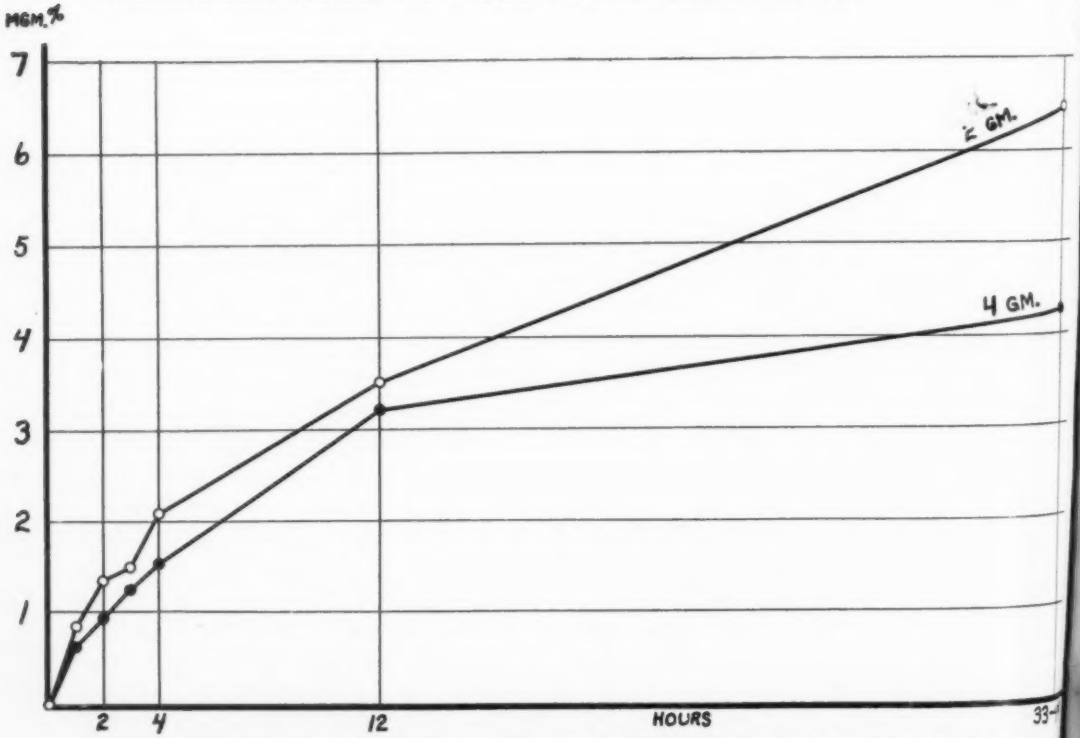

Fig. 3.—The blood concentration curves over a period of from thirty-three to forty-four hours after initial doses of 2 and 4 Gm., followed by a maintenance dose of 1 Gm. every four hours. The curves represent the median values of many determinations.

nonvomiting cases who received an initial dose of 4 Gm. At any rate, it seems to us that the figures are impressive enough to lead us to recommend a routine initial dose of 2 Gm. rather than the larger one of 4 Gm. The probability of vomiting will be lessened, and the blood concentration is likely to be not only as high but possibly even higher from the smaller dose.

It is interesting to speculate as to the cause of vomiting following administration of sulfapyridine. There are only two probable explanations: a local irritating action of the drug on the stomach, and a central action on the vomiting center. Both theories have their proponents. However, Marshall's demonstration that the sodium salt of sulfapyridine given intravenously caused vomiting,[2] is strong evidence for the central origin of the symptom.

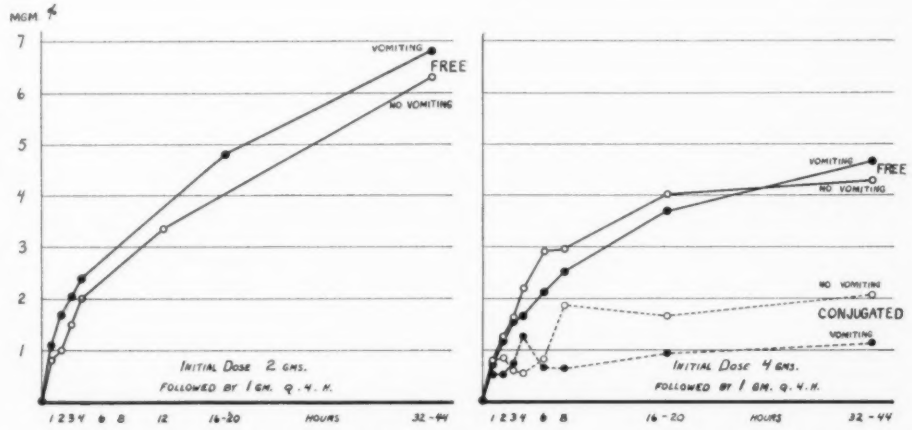

Fig. 4.—The relationship between vomiting and the concentration of free and of acetylated sulfapyridine in the blood. There were insufficient determinations of total sulfapyridine in the cases where the initial dose was 2 Gm. to compute the acetylated form.

The cause of the vomiting is not readily discerned from our investigations, but the fact that vomiting does not ensue following the initial large doses of 4 Gm. but only following subsequent smaller ones seems to us to argue against its purely gastric origin. Certainly a high concentration of the sulfapyridine in the blood is not by itself responsible, for with the higher concentrations following the smaller doses there is less probability of vomiting, as we have just noted. Moreover, the concentration during the period from twelve to forty-eight hours after the initial dose, when vomiting is most likely to occur, is about the same in the vomiting as in the nonvomiting cases when 4 Gm. have been given, and even higher for the vomiting than for the nonvomiting cases where 2 Gm. have been given. It has been suggested that the conjugated portion of the drug may be responsible for the vomiting. Our investigations do not bear this out, however, for as Fig. 4 shows, the level for the conjugated form is much lower where vomiting occurred than where it did not. One might consider the theory that perhaps there is a "critical vomiting level" for sulfapyridine in the blood were it not for the fact that the blood concentration at the time vomiting occurs varies tremendously in different individuals; however, had blood concentrations been determined at the time of onset of nausea, it is conceivable

that a fairly constant level might have been discovered. It has been our experience that frequently patients become nauseated or vomit early in the course of therapy, with relatively low blood levels; but if the drug is continued, the nausea lessens or disappears even while the blood concentration is rising. If vomiting is due to the action of the sulfapyridine in the blood on the vomiting center, it is probable that continued action by the drug eventually depresses the center after its initial stimulation.

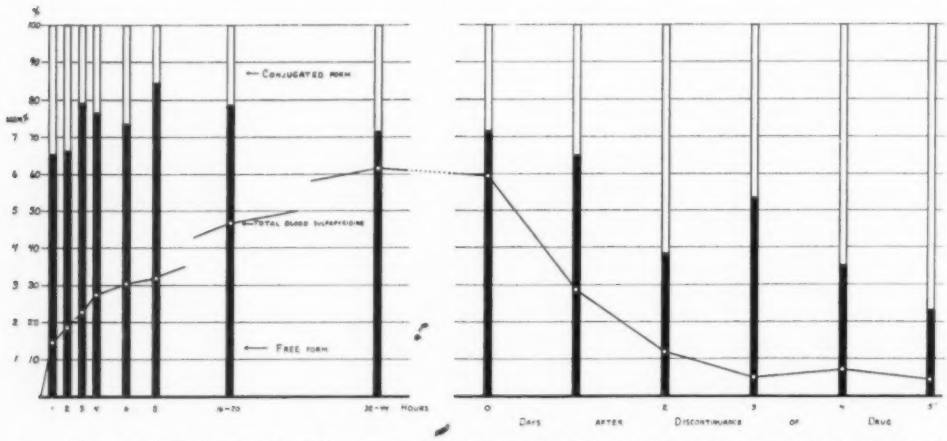

Fig. 5.—The blood concentration curve for total sulfapyridine throughout the stages of absorption, maintenance, and elimination, and the simultaneous relationship between the free and the acetylated forms. All the medians are from as many figures as were available.

(b) *Acetylation.* Acetylation or conjugation of the drug occurs very rapidly after it enters the blood stream—so rapidly that within an hour after it is given by mouth a very considerable proportion of it has already been conjugated (Fig. 5). In six or eight hours the maximum acetylation has occurred. During elimination the "free" form of the drug leaves the blood stream more rapidly than the "conjugated" form, so that during this phase the percentage of the acetylated form is much greater than at any other time. Table I shows this trend, the figures all representing median values and all following an initial dosage of 4 Gm.

TABLE I

THE ELIMINATION OF SULFAPYRIDINE: BLOOD CONCENTRATION STUDIES DURING THE ELIMINATION PHASE

	CONCENTRATION OF DRUG						
	ON DAY IT WAS STOPPED	DAYS LATER					
		1	2	3	4	5	
Free sulfapyridine	**4.23**	**1.85**	**0.45**	**0.26**	**0.25**	**0.10**	**mg. %**
Acetylated form	1.70	0.99	0.74	0.23	0.47	0.32	mg. %
Total sulfapyridine	5.93	2.84	1.19	0.49	0.72	0.42	mg. %

The degree of acetylation varies tremendously, however, not only from case to case but also from time to time in the same case. It may be as low as zero, or none at all, and as high as 80 per cent (exclusive of the "elimination stage," when 100 per cent of the drug remaining in the blood stream may be

in the conjugated form). In general, though, from 10 to 45 per cent is in the acetylated form, the average being about 33 per cent. We could find no evidence for the statement sometimes made that the degree of acetylation depends to some degree on the concentration of the drug in the blood stream.

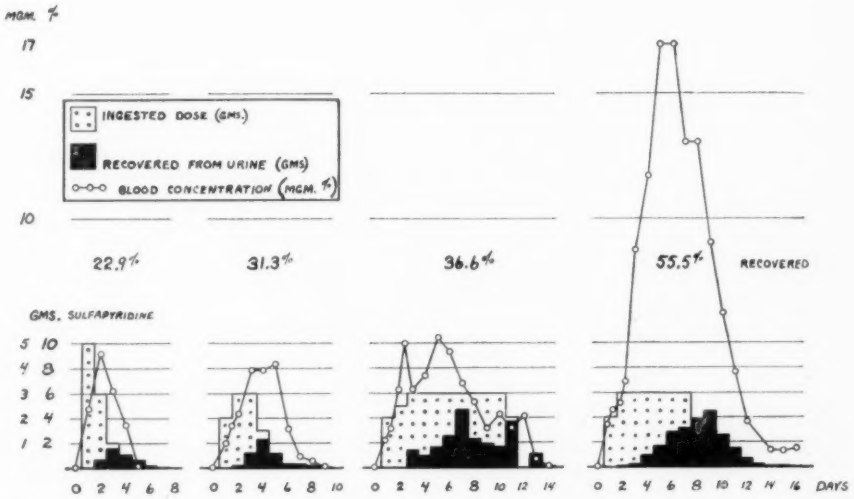

Fig. 6.—The relationship between the total amount of sulfapyridine ingested and the total amount recovered from the urine in 4 representative cases.

(c) *Elimination.* Following withdrawal of sulfapyridine by mouth, its elimination is fairly rapid (Fig. 5). Within three days most of it has been eliminated, but as long as five days after the last dose, easily measurable quantities can still be found in the blood stream. As noted above, the free form of the drug is excreted first and most rapidly, the conjugated form being excreted more slowly. Its recovery from the urine tends, as might be expected, to follow the blood level quite closely. After the drug is discontinued, the amount recovered from the urine parallels the diminishing blood level, so that it disappears from the urine at about the same time it does from the blood stream (Fig. 6). We have not noted the lag between the disappearance from the blood and from the urine spoken of by others. Generally speaking, when the blood level falls to about 0.4 mg. per 100 c.c., no more appreciable quantities can be recovered from the urine.

In 16 cases it was possible to collect the entire amount of urine passed during the whole course of sulfapyridine therapy. The amount of drug in the urine was measured quantitatively for the entire period. Table II records the findings. From this table it will be seen that the proportion of ingested drug which was recovered from the urine varied from 21.6 per cent to 55.5 per cent, the average being 29.8 per cent. Interestingly, when the cases are subdivided into vomiting and nonvomiting groups, the average excretion was still 29.8 per cent for both groups. It is presumed that the balance not recovered was not absorbed but passed unchanged through the gastrointestinal tract or was vomited. Hence, in general, one may say that about 30 per cent of the ingested drug is absorbed. In general, the free and the acetylated forms oc-

cur in the urine in about equal proportions; where there is vomiting, however, the free form is proportionately much lower (42.5 per cent) than where there is no vomiting (64.0 per cent), although the reverse is true for its distribution in the blood stream.

TABLE II

THE ELIMINATION OF SULFAPYRIDINE: QUANTITATIVE URINE STUDIES AND COMPARISON WITH AMOUNT OF DRUG INGESTED

CASES	AMOUNT RECOVERED FROM URINE			TOTAL ORAL DOSE	INGESTED DOSE RECOVERED
	FREE	PER CENT OF TOTAL	TOTAL		
Vomiting	Gm.		Gm.	Gm.	Per cent
12	10.40	46.8	22.20	40	55.5
16	13.11	64.5	20.31	73	27.8
18	15.25	54.3	28.07	94	29.8
19	2.02	56.6	3.57	12	29.8
20	2.20	60.1	3.66	13	28.2
29	2.26	38.1	5.94	18	33.0
34	5.63	25.2	22.33	61	36.6
36	1.68	28.2	5.95	19	31.3
40	1.71	18.8	9.08	42	21.6
41	1.10	26.6	4.13	18	22.9
No vomiting					
8	5.87	66.2	8.87	38	23.3
9	3.18	86.0	3.70	16	23.1
10	3.34	52.4	6.37	20	31.9
17	7.18	69.2	10.38	28	37.1
26	3.92	61.8	6.34	16	39.6
35	21.72	41.7	52.07	188	27.7
Median for vomiting group					29.8
Median for no vomiting group					29.8
Median for entire group					29.8

(d) *The optimum concentration of sulfapyridine.* Theoretically, it might be assumed that to obtain 100 per cent cures of pneumonia, the blood concentration should be above a certain figure, all other conditions being equal. Practically everyone who has written on this subject has reported a certain number of deaths in his series—not as many, it is true, as following other types of treatment, but still some deaths. One naturally asks if it is not probable that many, if not most, of those deaths were avoidable and were due to the fact that the concentration of sulfapyridine in the blood was too low to be effective. No figures concerning this point seem to be available. We have analyzed our own figures with this in mind.

In 103 cases of pneumonia of various kinds treated in the Louisville hospitals during the past season there were 6 deaths—a mortality rate of slightly under 6 per cent. These 6 cases include one patient who had advanced cardiorenal disease and who was already living "on borrowed time." It was not reasonable to expect any beneficial effects from sulfapyridine in this patient, but, nevertheless, she was given the benefit of the doubt and the case is included in this series. Two of the remaining 5 cases had extensive, bilateral bronchopneumonia, and the remaining 3 had lobar pneumonia.

The highest concentration of free sulfapyridine in the blood *at any time* in the fatal cases is compared with the highest concentration in the recovered cases in Table III, the figures being grouped over a 2 mg. per cent range. In

60 per cent of the fatal cases the concentration was less than 4 mg. per cent, and in 80 per cent, it was less than 6 mg. per cent, whereas in only 30 per cent of the recovered cases was the greatest concentration under 4 mg. per cent; 30 per cent had a maximum concentration between 4 and 6 mg. per cent, and 40 per cent had a maximum concentration over 6 mg. per cent. The obvious implication is that, although it is reasonable to expect a certain number of cures with a blood concentration of free sulfapyridine below 6 mg. per cent, yet to ensure the maximum number the concentration may well be *over* 6 mg. per cent.

TABLE III

DISTRIBUTION OF THE MAXIMUM CONCENTRATION FIGURES IN ALL CASES OF PNEUMONIA WHERE THERE WERE AT LEAST 3 DETERMINATIONS (FREE SULFAPYRIDINE)

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Recovered cases (57)	17 29.8%	17 29.8%	13 22.8%	10 17.6%
Fatal cases (5)*	3 60.0%	1 20.0%	1 20.0%	0 0%

*One of the fatal cases had unreliable determinations.

A fairer method of arriving at the optimum figure is to compare the maximum concentration in the fatal cases with that concentration in the recovered cases, *following which* the temperature remained normal and the patient went on to recovery, for in a few cases the blood concentration continued to rise even after the patient was getting well. In Table IV is recorded this comparison, the figures being based on as many cases as we had available for the preparation of the data needed. Here too, in only 30 per cent of the recovered cases was the concentration below 4 mg. per cent, about 30 per cent had a concentration between 4 and 6 mg. per cent, and 40 per cent had a concentration over 6 mg. per cent. These figures compare almost exactly with those in which the *maximum* concentration is taken because of the fact that it was in *only a few* cases that the concentration kept rising after the patient was on the road to recovery.

TABLE IV

COMPARISON OF THE MAXIMUM CONCENTRATION IN THE FATAL CASES OF PNEUMONIA, WITH THAT CONCENTRATION FOLLOWING WHICH THE TEMPERATURE REMAINED NORMAL AND THE PATIENT WENT ON TO RECOVERY

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Recovered cases (22)	7 31.8%	6 27.3%	7 31.8%	2 9.1%
Fatal cases (5)*	3 60.0%	1 20.0%	1 20.0%	0 0%

*One of the fatal cases had unreliable determinations.

We may reiterate, then, that from our work it seems desirable, in order to reduce the mortality rate to the lowest possible figure, that the blood concentration of free sulfapyridine be maintained around at least 6 mg. per cent. It follows from this that in actual practice fairly frequent determinations of blood concentrations should be made, especially if the patient does not seem to be responding to treatment. If the concentration is not reaching a level of at least

6 mg. per cent and the patient is not improving, then the drug should be given parenterally, or the sodium salt should be used, whenever it becomes commercially available. While this precaution (frequent checks on the blood concentration) may seem a useless expense to the patient and may as a matter of fact actually not be necessary in the great majority of cases, yet for the sake of the exceptional cases it seems to us desirable to subject every patient to this routine. Our position in this respect seems justified since in the human being we are not yet as cognizant as we are in mice of the effectiveness of the drug in the various types of the pneumococcus.

TABLE V

COMPARISON OF CASES WITH AND WITHOUT BACTERIEMIA. THE DISTRIBUTION OF THE BLOOD CONCENTRATION FIGURES FOLLOWING WHICH THE TEMPERATURE REMAINED NORMAL AND THE PATIENTS WENT ON TO RECOVERY

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Positive blood culture (11)	4 **36.4%**	2 **18.2%**	5 **45.5%**	0 **0%**
Negative blood culture (11)	3 **27.3%**	4 **36.4%**	2 **18.2%**	2 **18.2%**

*One patient had a *Staph. aureus* blood culture; the remainder had pneumococci.

TABLE VI

COMPARISON OF CASES WITH AND WITHOUT BACTERIEMIA. THE DISTRIBUTION OF FIGURES OF MAXIMUM CONCENTRATION AT ANY TIME

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Positive blood culture (16)*	2 **12.5%**	4 **25.1%**	5 **31.3%**	5 **31.3%**
Negative blood culture (19)	7 **36.9%**	6 **31.6%**	4 **21.1%**	2 **10.5%**

*Two patients had *Staph. aureus* blood cultures; the remainder had pneumococci.

Bacteriemia.—In an effort to determine the effects of different blood concentrations in cases with and without a blood stream infection, our figures were analyzed as outlined immediately above, the cases being divided into recovered cases with and without positive blood cultures. (Note: Of the fatal cases, 3 had positive blood cultures, 1 had a negative culture, and in 2 no blood was taken for culture.) From Table V can be seen the paradoxical fact that 55 per cent of the patients with bacteriemia had a normal temperature followed by recovery, even though the blood level was under 6 mg. per cent, whereas 64 per cent of the nonbacteriemic patients recovered under the same circumstances. On the other hand, when one considers the maximum concentration at any time (Table VI), it is seen that only 40 per cent of the bacteriemic patients recovered when the maximum concentration was less than 6 mg. per cent, whereas about 70 per cent of the nonbacteriemic patients recovered under the same conditions. From this it follows that it was perhaps not a bad thing that often the blood concentration kept on rising after the patient began to get well, for had it not he might not have recovered. These findings do not contradict our opinion as to a minimum blood level of 6 mg. per cent being desirable.

SUMMARY AND CONCLUSIONS

1. In the Louisville hospitals sulfapyridine has been given to a total of 131 patients, of whom 103 had pneumonia. Of these, extensive studies were made concerning absorption, concentration in the blood, and excretion of the drug in 46 patients, of whom 38 had pneumonia.

2. The drug was administered orally in three ways: an initial dose of 2 Gm. followed by 1 Gm. every four hours; an initial dose of 4 Gm. followed four hours later by 1 Gm. every four hours; an initial dose of 4 Gm. followed eight hours later by 1 Gm. every four hours.

3. Absorption is very erratic, the height of the absorption curve being unpredictable from identical doses and from patient to patient, and there being no correlation between the size of the dose and the height of the blood concentration curve. In general, the blood concentration is higher following a dose of 2 Gm. than of 4 Gm.

4. Vomiting occurs in about 60 per cent of the cases, but is more frequent (67 per cent) following an initial dose of 4 Gm. than of 2 Gm. (45 per cent). For this reason and from the observation noted above, we recommend the routine employment of an initial dose of 2 Gm. rather than larger doses. Our work does not clarify the cause of the vomiting, but we feel that it is of central and not of gastric origin. Certainly it is not due to an increased concentration of the acetylated form, for in the vomiting cases the acetylated form of the drug was much lower than in the cases where there was no vomiting. It is likewise difficult to explain, on the basis of vomiting, why the concentration should be higher from the smaller dose.

5. Acetylation or conjugation of the drug occurs very rapidly in the body, about 35 per cent of it being acetylated within the first hour. Throughout the course of administration about 33 per cent acetylation is present, although the degree of acetylation varies from 0 to 100 per cent. During the elimination phase the degree of acetylation is much greater than at any other time. We found no evidence that the degree of acetylation bears any relation to the total concentration of drug in the blood.

6. After the withdrawal of the drug by mouth its elimination occurs rapidly at first, then more slowly, so that as long as 5 days later appreciable quantities can still be found in the blood and in the urine. The free form is eliminated more rapidly than the acetylated form. The drug disappears from the urine at about the same time it does from the blood. Quantitative determinations show that from 21 to 56 per cent of the drug taken by mouth can be recovered from the urine, the average being 30 per cent.

7. Of our 103 patients with pneumonia, 6 died. Of these 6 it was reasonable to hope for recovery in 5; of these 6, 60 per cent had a maximum blood concentration less than 4 mg. per cent and 80 per cent less than 6 mg. per cent. From this it is concluded that had it been possible to have raised the concentration in all the fatal cases to over 6 mg. per cent, some of them might have recovered. From this we make the generalization that in order to ensure the maximum recovery rate, frequent blood concentration studies should be made

in *every* case of pneumonia, and if the patient does not seem to be doing well and the blood concentration is below 6 mg. per cent, then the drug or its sodium salt should be administered parenterally.

8. Only 40 per cent of the cases with bacteriemia recovered with a blood concentration less than 6 mg. per cent, whereas in the cases with a negative blood culture, 70 per cent recovered under the same conditions.

We are indebted to Miss Beatrice Denny for her technical assistance.

REFERENCES

1. Marshall, E. K., Jr., and Litchfield, J. T., Jr.: The Determination of Sulfanilamide, Science **88**: 85, 1938.
2. Marshall, E. K., Jr., and Long, Perrin H.: The Intravenous Use of Sodium Sulfapyridine, J. A. M. A. **112**: 1671, 1939.

STUDIES OF SULFANILAMIDE IN BLOOD AND URINE OF RABBITS INFECTED WITH BETA HEMOLYTIC STREPTOCOCCI*

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SINCE the observation that sulfanilamide and related compounds are efficacious in the treatment of various infections, particularly those caused by the beta hemolytic streptococcus, many investigations have been carried out to determine the properties and actions of these drugs. At the time the present work was undertaken, however, none of the investigators had established a definite correlation between the dose of the drug and its level in the blood stream which should be maintained for clinical cure. The present investigation sought to supply this information, as well as to study the elimination of the sulfanilamide in the urine.

Infection was induced in rabbits by injecting beta hemolytic streptococci subcutaneously. Various methods of inoculation were tried and discarded because they failed to produce satisfactory foci with bacteriemia in the animals. The method finally chosen for the experiments utilized a mixture of 5 c.c. of an eighteen-hour blood broth culture and an equal volume of a 5 per cent preparation of mucin in salt solution. Two cubic centimeters of this suspension were injected subcutaneously through the shaved skin of the abdomen.

Considerable difficulty was experienced in producing a bacteriemia. Although the local lesions measured as much as 140 by 90 mm. and were elevated as much as 25 mm. and although they persisted two to three weeks, only 2 of 28 rabbits showed any streptococci in the blood stream. Since the disappearance of the bacteriemia could not be used as a criterion of successful

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treatment, we had to rely on the healing of the local lesion, return of temperature to normal, and recovery of the animal to adjudge the success of treatment.

Rectal temperatures were recorded twice daily and blood cultures were made daily until the animal died or recovered. Two cubic centimeters of blood were taken from the heart twice daily, the one at approximately the time when the blood level of the drug would be at the maximum, and the other at the time when this level would be at the minimum. On each of these specimens the amount of free and total sulfanilamide was determined by a method devised by Marshall.[1] In each of 7 sets of experiments 4 rabbits (weighing about 2 kg.) were studied; 2 animals were treated and 2 served as controls. The former were placed in metabolism cages and the urine was collected for twenty-four-hour periods. Determinations of free and total sulfanilamide were made on these urine specimens as long as the animals were under treatment. The method of analysis was that of Marshall, Emerson, and Cutting,[2] as modified by one of us (Hansen[3]).

For treatment, sulfanilamide was prepared in 0.25, 0.5, and 1 per cent solutions. Each of the treated rabbits in a series received 100 c.c. of one of these solutions twice in twenty-four hours through a stomach tube. The first daily dose was given in the morning, followed by the second dose about four hours later. The next treatment was not given until the next morning, twenty hours later.

Available data from studies on man, dogs, rabbits, rats, and mice[2, 4-8] indicate considerable differences regarding the time of administration of the drug and the attainment of a maximum blood level between human beings and lower animals, as well as between individual members of the same species. Furthermore, the free sulfanilamide is presumed to be more active therapeutically than the acetylated derivative. Obviously, it was necessary to obtain information relative to the maximum level of the sulfanilamide in the blood and also data concerning its conjugation before studying infected animals. Four series of uninfected rabbits were treated orally over periods of three days. Blood analyses were made as indicated below (Part I). This information was then used in choosing the time for taking blood specimens for study from the infected animals (Part II).

PART I. LEVEL OF FREE AND CONJUGATED SULFANILAMIDE IN BLOOD OF UNINFECTED RABBITS

Two rabbits were treated orally with 100 c.c. of a 1 per cent aqueous solution of sulfanilamide at 8 A.M. on two consecutive days. Blood specimens were obtained at regular intervals and on the morning of the third day. Curves I and II in Fig. 1 give the blood levels of the total and free sulfanilamide in these rabbits on the two consecutive days, and Curve III indicates the per cent of the conjugated drug. The points of interest are: (1) On both days and for both rabbits the maximum level of the drug (free and total) was obtained with the first blood specimen, two hours after the dose, and these values were approximately the same for both rabbits and were nearly equal on both days.

(2) The levels of the total and free sufanilamide ran nearly parallel, dropping slowly during the first eight hours after the dose and falling to approximately one-tenth of the maximum value twenty-four hours after the dose. (3) The conjugated drug quickly reached a level which remained nearly constant during the first eight hours. (4) The trend of conjugation was decidedly different for the two rabbits; in rabbit 14 the rate of conjugation was slow, and in rabbit 15 it was comparatively rapid. This difference in the conjugation was clearly indicated two hours after the dose, but more noticeably after greater time intervals, so that at the end of eight hours the first rabbit had about 70 per cent of the drug in the free state, while the other had about 70 per cent conjugated. (5) Twenty-four hours after the dose the percentage of the relatively small amount of free and conjugated drug still in the blood was about the same for both rabbits. On the basis of these observations it would seem best to obtain blood specimens for maximum values two hours after the dose, or possibly sooner than this, but about two to four hours after the dose to show the trend of conjugation.

TABLE I

SULFANILAMIDE CONCENTRATION IN BLOOD OF NORMAL RABBITS ON THE THIRD DAY OF TREATMENT

(100 ml. of 1 per cent solution of sulfanilamide administered twice daily.)

TIME	HOURS AFTER DOSE	RABBIT 14					RABBIT 29				
		MG. PER 100 ML. OF BLOOD			PER CENT		MG. PER 100 ML. OF BLOOD			PER CENT	
		TOTAL S	FREE S	CONJ. S	FREE S	CONJ. S	TOTAL S	FREE S	CONJ. S	FREE S	CONJ. S
7:00 A.M.	19	43.5	32.2	11.3	74.0	26.0	7.0	1.35	5.65	19.3	80.7
8:00	1	60.6	51.3	9.3	84.3	15.7	29.8	23.8	6.0	79.9	20.1
9:00	2	52.6	43.5	9.1	82.7	17.3	29.8	22.0	7.8	73.8	26.2
10:00	3	54.8	42.5	12.3	77.6	22.4	27.0	19.4	7.6	71.8	28.2
12:00 M.	5	50.0	39.2	10.8	78.5	21.6	23.25	15.93	7.32	68.6	31.4
1:00	1	57.1	50.0	7.1	87.5	12.5	42.5	36.4	6.1	85.6	14.4
2:00	2	57.1	46.5	10.6	81.2	18.6	40.8	32.2	8.6	79.0	21.0
3:00	3	57.1	50.0	7.1	87.5	12.5	35.1	29.0	6.1	82.6	17.4
5:00	5	54.0	44.5	9.5	82.4	17.6	28.6	22.2	6.4	77.7	22.4
10:00 A.M.	22	-	-				2.42	Trace	2.42	0.0	100.0

However, in order to secure data, first, regarding the blood level and conjugation of sulfanilamide obtained with different dosages, and secondly, concerning the increase of the maximum blood level when the drug is administered more than once a day, 100 c.c. of a 1 per cent aqueous solution of sulfanilamide was administered by stomach tube twice a day on three consecutive days to several rabbits. Two animals were treated in the same manner with 0.25 per cent solution. Blood specimens were taken at regular intervals on the third day of treatment.

Table I presents data on two of three rabbits which received the 1 per cent solution. Several points are to be noted. (1) The blood level in rabbit 14 was very high on the morning of the third day of treatment, nineteen hours after the preceding dose, especially when compared with the values obtained with the same rabbit some three weeks earlier, when the level after single

doses dropped to from 2 to 3 mg. for total sulfanilamide (Fig. 1) and likewise when it is compared with rabbit 29 that received identical treatment. (2) After each administration of the drug the highest values for the total and free sulfanilamide were found in the first specimen of blood, one hour after the dose. (3) The increase in level for total drug was about the same for both rabbits after the first dose, and after the second dose it was again similar for one rabbit but much less for the other. (4) A new high value was observed for the blood level of the drug in one of the rabbits after the second administration of the drug. (5) The drop in the blood level was not very rapid in

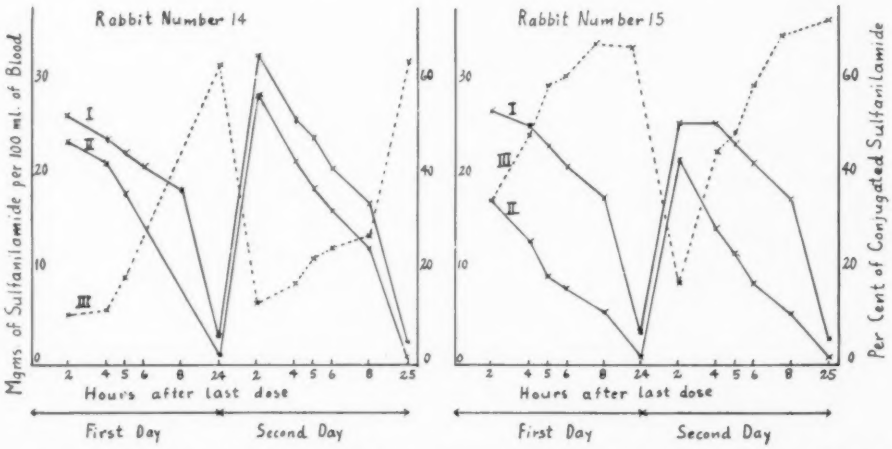

Fig. 1.—Sulfanilamide concentration in the blood of normal rabbits (100 ml. of a 1 per cent solution of sulfanilamide administered daily on two consecutive days). Curve I = Total sulfanilamide. Curve II = Free sulfanilamide. Curve III = Per cent of conjugated sulfanilamide.

the first five hours after administration of the drug. (6) The trend to conjugation was comparatively slight for both rabbits, but it was well established at the end of the third hour after a dose. It appears to be a consistent quality of a given animal: rabbit 14, for example, had manifested a similar capacity for conjugation when it was used before. Finally, the excretion appears to be unimpaired in one of the rabbits, and conjugation is complete for the drug which still remains in the blood twenty-two hours after the last dose.

Almost all the points enumerated hold also for the two rabbits which received the 0.25 per cent solution. The level for total drug appears to be roughly proportional to the dose. The highest levels were found in the first blood specimen, one hour after the dose. The trend to conjugation again was clearly indicated three hours after a dose, but conjugation was much higher than in the former set of rabbits, which indicated a characteristic difference between individual rabbits. The level for the total drug in the blood dropped slowly during the first five hours after a dose, but was down to approximately 1 mg. per cent for the total drug and only a trace of free drug twenty-two hours after the last dose.

In view of the fact that sulfanilamide is generally administered to man in the solid form and that it has a relatively low solubility, the drug was given

in gelatin capsules for a comparison with administration in solution. The drug was administered in divided doses at the rate of 1 Gm. per kilogram of body weight, twice daily, and on three consecutive days to 5 rabbits. Blood specimens were obtained at regular intervals for analysis on the first and third days and in addition on the morning of the second and fourth days of the experiment. Data from two of these rabbits are presented in Fig. 2.

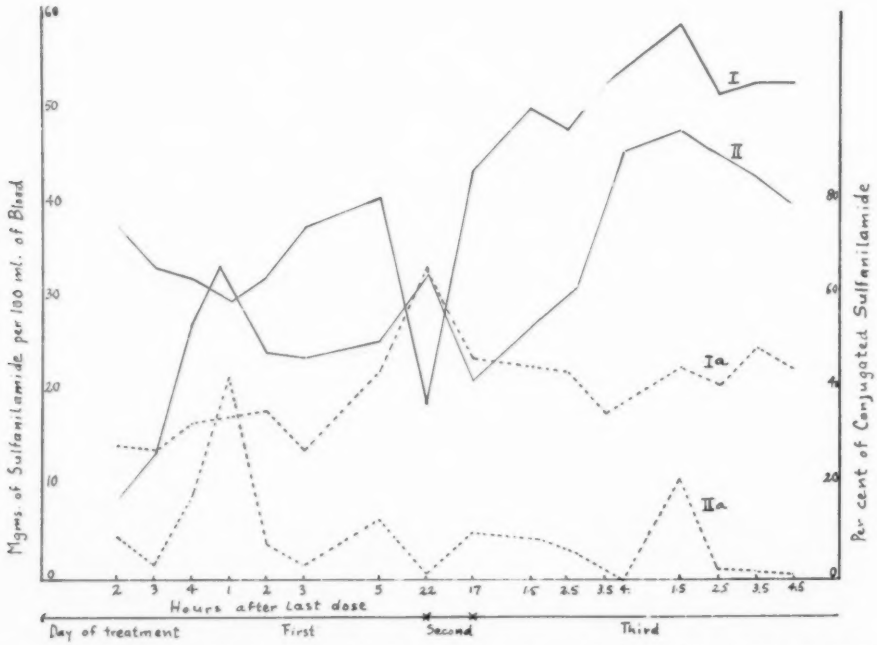

Fig. 2.—Sulfanilamide in the blood of normal rabbits. Administration in gelatin capsules twice daily on three consecutive days (1 Gm. of sulfanilamide per kilogram of body weight per day). Curves I and II = Total sulfanilamide for rabbits I and II. Curves Ia and IIa = Per cent of conjugated sulfanilamide for rabbits I and II, respectively.

The most characteristic feature to be noted in this series, as contrasted with administration of the drug in solution, was a marked irregularity in obtaining a maximum blood level. The actual level reached was very high, especially on the third day. In fact, there appeared to be a definite retention of the drug in both rabbits, judged on the basis of a high general level, and unusually high values for the morning specimens on the second, third, and fourth days, and on the recovery of the drug in the urine of one of the rabbits, which was 25.3, 35.8, and 30.6 per cent of the intake on successive days of treatment. Each of the rabbits appeared to have its own characteristic conjugation, the one having a high and the other a distinctly low capacity.

One of the rabbits was very ill and died at the end of the third day of treatment. Death was probably due to the effect of the drug. The other rabbit survived, although the blood level of the drug in this rabbit was markedly higher than in the rabbit that died. It seems that administration of the drug in solution results in a more uniform handling of the drug; it is also to be noted that in rabbits there may be an accumulation of the drug, which

may be a result of a temporary impairment of kidney function, a phenomenon we have repeatedly observed in infected rabbits treated with sulfanilamide.

SUMMARY AND CONCLUSIONS

1. Levels and distribution of free and conjugated sulfanilamide were studied in the blood of four groups of uninfected rabbits in order to determine maximum blood levels and trends to conjugation of the drug.

2. Maximum blood levels were obtained within one hour after administration of the drug, when it was given in an aqueous solution by stomach tube. But when the solid form was administered in gelatine capsules, maximum blood levels were obtained at irregular intervals after a dose.

3. The ability to conjugate the drug varies greatly with different rabbits. It appears to be characteristic for a given animal and is definitely established two to three hours after administration of the drug.

4. Blood levels of the drug increase with each administration and are roughly proportional to the size of the dose when the drug is administered in aqueous solution.

5. The amount of the drug in the blood drops slowly during the first five hours after a given dose.

PART II. SULFANILAMIDE IN BLOOD AND URINE OF RABBITS INFECTED WITH HEMOLYTIC STREPTOCOCCI

Seven series of rabbits were studied, each group consisting of four animals. They were infected and treated as previously described. Blood specimens were obtained twice daily by cardiac puncture: morning specimens, eighteen to twenty hours after the previous dose, to give minimum blood levels of the drug; and evening specimens, two and one-half and, in some instances, four and one-half hours after the last dose to give maximum blood level values, and also to indicate the tendency to conjugation. Elimination of the drug was determined by quantitative urinalysis. The drug was administered at the rate of 0.5 Gm. per day (approximately 0.25 Gm. per kilogram of body weight), 1 Gm. per day (about 0.5 Gm. per kilogram of body weight) in two series and 2 Gm. per day (about 1 Gm. per kilogram of body weight) in four series (Table II).

Treatment With 0.5 Gm. of Sulfanilamide Per Day.—In series[1] one of the controls and one of the treated animals recovered (Table II). The blood and urine data are given in Table III for the two treated rabbits over a period of eight days. For rabbit 37 the blood values for total and free sulfanilamide are given over a period of eighteen days (Fig. 3). This represents the first two periods of treatment. Rabbit 39 survived eight days, and rabbit 37 recovered after daily treatment over a period of four weeks.

Comparison of data shows similarities and differences which suggest certain conclusions with regard to effectiveness and toxicity of the drug. Elimination of the drug in the urine was nearly identical throughout the period of observation. The percentages of the conjugated form of the drug in the urine were almost identical. Likewise, the percentage of conjugated drug was uniformly

TABLE II

RESULTS OF HEMOLYTIC STREPTOCOCCAL INFECTIONS IN RABBITS

DOSE PER DAY	SERIES	TREATED				UNTREATED		
		RABBIT NO.	DAYS TREATED	DAYS SURVIVED	CAUSE OF DEATH AND BACTERIOLOGIC FINDINGS	RABBIT NO.	DAYS SURVIVED	BACTERIOLOGIC FINDINGS AT AUTOPSY
0.5 Gm.	I	37	20	*	*	38	1	Local lesion—Hemolytic streptococci Blood—Hemolytic streptococci
		39	7	8	Drug Local lesion—Hemolytic streptococci	40	*	*
1.0 Gm.	II	34	2	7	Drug Local lesion—Hemolytic streptococci	33	*	*
		36	2	*	*	35	10	Local lesion—Hemolytic streptococci Liver abscess—Hemolytic streptococci
	III	41	17	*	*	43	*	*
		42	14	*	*	44	*	*
2.0 Gm.	IV	4	4	4	Accidental death during treatment	14	*	*
		10	5	7	Drug Local lesion—Hemolytic streptococci	15	*	*
	V	20	1	3	Peritonitis Local lesion—Hemolytic streptococci Peritoneal exudate Hemolytic streptococci	19	21	Local lesion—Hemolytic streptococci
		25	24	*	*	21	21	Local lesion—Hemolytic streptococci
	VI	24	18	37	Drug Cultures—No growth	27	30	Local lesion—Hemolytic streptococci
		26	7	10	Drug Local lesion—Hemolytic streptococci	28	*	*
	VII	29	11	*	*	31	4	Local lesion—Hemolytic streptococci
		30	5	8	Drug Local lesion—Hemolytic streptococci	32	2	Multiple coccidial abscesses in liver Local lesion—Hemolytic streptococci

*Recovered.

TABLE III

SULFANILAMIDE IN BLOOD AND URINE OF RABBITS: STREPTOCOCCUS INFECTION
(Intake of drug 0.5 Gm. per day = approximately 0.25 Gm. per kg. of body weight.)

DAYS TREATED	TIME OF BLOOD SPECIMEN	RABBIT 37 (RECOVERED) SULFANILAMIDE IN BLOOD TOTAL MG. PER 100 ML.	RABBIT 37 (RECOVERED) SULFANILAMIDE IN BLOOD PER CENT CONJUGATED	RABBIT 37 (RECOVERED) SULFANILAMIDE IN URINE COLLECTION PERIOD HOURS	RABBIT 37 (RECOVERED) SULFANILAMIDE IN URINE RECOVERY OF INTAKE PER CENT	RABBIT 37 (RECOVERED) SULFANILAMIDE IN URINE PER CENT CONJUGATED	RABBIT 39 (DIED) SULFANILAMIDE IN BLOOD TOTAL MG. PER 100 ML.	RABBIT 39 (DIED) SULFANILAMIDE IN BLOOD PER CENT CONJUGATED	RABBIT 39 (DIED) SULFANILAMIDE IN URINE COLLECTION PERIOD HOURS	RABBIT 39 (DIED) SULFANILAMIDE IN URINE RECOVERY OF INTAKE PER CENT	RABBIT 39 (DIED) SULFANILAMIDE IN URINE PER CENT CONJUGATED
1	A.M.*										
	P.M.	7.98	40.2	24	57.8	84.1	10.13	26.8	24	–	–
2	A.M.	4.04	21.0				0.92	7.6			
	P.M.	7.73	60.2	24	72.5	89.3	5.27	39.3	24	60.9	89.4
3	A.M.	1.85	100.0				4.24	78.0			
	P.M.	7.94	63.0	24	77.5	81.8	8.93	67.0	24	75.6	88.0
4	A.M.	2.72	100.0				5.06	84.0			
	P.M.	9.75	67.6				13.00	70.7			
5	A.M.	No treatment					No treatment				
	P.M.			48	108.4	87.6			48	98.9	88.1
6	A.M.	3.20	20.6				4.39	100.0			
	P.M.	12.5	69.3	24	44.5	86.2	8.20	41.6	24	44.2	86.3
7	A.M.	5.43	51.0				6.10	84.8			
	P.M.	9.26	64.0	24	65.9	88.5	14.00	66.4	24	65.4	84.5
8	A.M.	2.91	66.0				12.28	81.7			
	P.M.	9.50	53.5	24	65.1	87.0	17.55	65.8	?	24.2	83.6
9	A.M.	4.2	60.0				Rabbit died during the night				

*A.M. = morning specimen, eighteen to twenty hours after last dose (minimum blood level).
P.M. = evening specimen, two and one-half hours after last dose (maximum blood level).

higher in the urine than in the blood, which indicates that the conjugated form is more readily eliminated than the free drug. This is also true in human beings.[3, 9] They were similar in requiring two to three days to reach an approximately even level for percentage elimination of drug intake, which is explained by retention in the tissues during the initial treatment.[2, 3] That this is true is substantiated by further urine data from both animals. In a forty-eight-hour period elimination with intake limited to the first twenty-four hours, elimination is higher than the previously established level and even in excess of the intake. This indicates a drainage of the drug out of the tissues. On the succeeding days of treatment this is followed first by a lowered elimination rate, indicating retention by the tissues, and then by an increased elimination approximating the former rate. These similarities indicate that the kidneys functioned efficiently in both animals.

The picture of the blood was not so uniformly similar. The trend to conjugation tended to be higher for rabbit 39 than for rabbit 37. The amount of drug in the conjugated form was also generally higher, especially during the last several days, when it rose to above 10 mg. per cent in rabbit 39, while in rabbit 37 it seldom rose above 5 mg. per cent nor did the blood picture in rabbit 37 change appreciably during the later period of observation (Fig. 3). The difference in the amount of free drug in the blood was not marked at any time. On the whole then, while the amount of total drug was higher in rabbit 39 than in rabbit 37, especially during the last several days of its life, this difference was due essentially to a higher level of the conjugated drug.

It appears that the drug level in rabbit 37 was adequate for the control of infection, while in rabbit 39, in spite of a higher total drug level, due principally to a greater amount of the conjugated form of the drug, the infection could not be controlled. At autopsy the blood was sterile, but hemolytic streptococci were cultured from local lesions. It may be that toxins entered the circulation from the focus of infection and that death was due to toxemia. However, it seems to us that the toxic effect of the greater amount of the drug in the circulation, and especially of the conjugated form, was definitely a contributory factor in causing death.

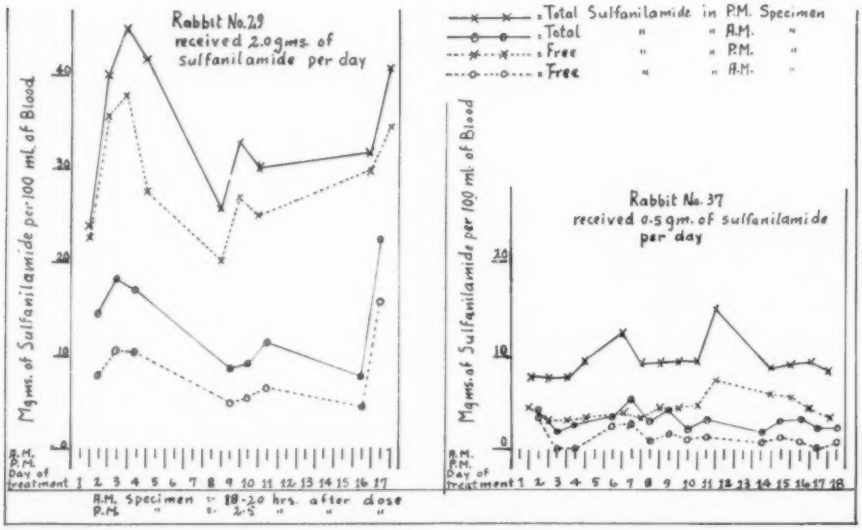
Rabbit No. 29
received 2.0 gms. of
sulfanilamide per day
Total Sulfanilamide in P.M. Specimen
Total " " A.M. "
Free " " P.M. "
Free " " A.M. "
Mgms. of Sulfanilamide per 100 ml. of Blood
Rabbit No. 37
received 0.5 gm. of sulfanilamide
per day
Day of treatment 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
A.M. Specimen = 18-20 hrs. after dose
P.M. " = 2-5 " " "

Fig. 3.—Maximum and minimum blood levels of total and free sulfanilamide in rabbits infected with beta hemolytic streptococcus.

Treatment With 1.0 Gm. of Sulfanilamide Per Day.—Two series were used in this group. In the first of these, series II, one of the controls and one of the treated animals recovered. The control was definitely infected as judged by temperature and lesion. In the other rabbit, infection was apparently localized since the blood was sterile and the temperature became normal after two days of treatment. The blood level was high on the first day of treatment, 18.2 mg. per cent, but dropped to about 4 mg. per cent, a level lower than in the preceding series in which only half as much drug was administered. Conjugation and elimination were comparable to what was found in the preceding series. The amount of the conjugated drug in the blood was above 5 mg. per cent in only the first specimen. The death in the other control was no doubt due to the infection, as indicated by the temperature, the nature of the lesion, and the detection of streptococci in the lesion and in a liver abscess. The other treated animal died after two days of treatment. Streptococci were found in the lesion but not in the blood. However, it is probable that the progressive accumulation of the drug in the blood, rising to

about 32 mg. per cent on the second day, had a severely toxic effect on the animal and probably was a contributory factor in causing death. The amount of conjugated drug in the blood was only about 5 per cent of the total, a very low conjugation, but in the urine it rose to an average of 25 per cent of the total.

In the other series of this group, series III, all the animals recovered. The two treated animals, rabbits 41 and 42, recovered after treatment over a period of three weeks.

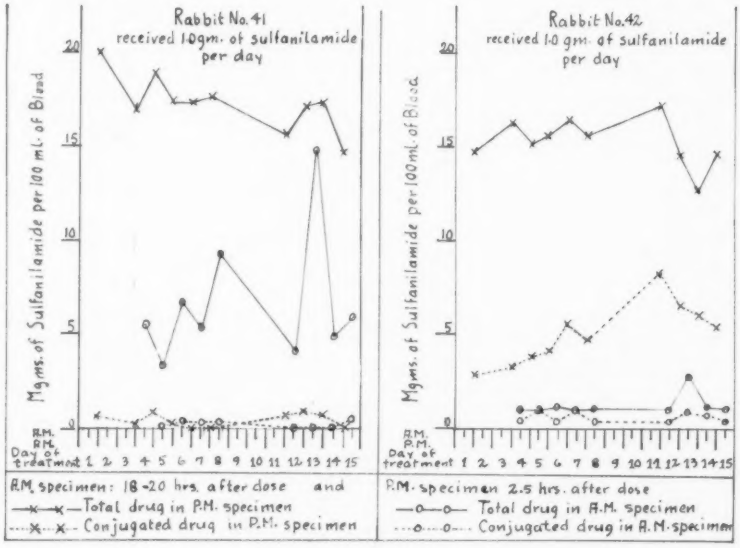
Rabbit No. 41
received 1.0gm. of sulfanilamide per day
Mgms. of Sulfanilamide per 100 mL. of Blood
Day of treatment
A.M.
P.M.
A.M. specimen: 18-20 hrs. after dose and
Total drug in P.M. specimen
Conjugated drug in P.M. specimen
Rabbit No. 42
received 1.0 gm. of sulfanilamide per day
A.M. specimen 2.5 hrs. after dose
Total drug in A.M. specimen
Conjugated drug in A.M. specimen

Fig. 4.—Maximum and minimum blood levels of total and conjugated sulfanilamide in rabbits infected with beta hemolytic streptococcus.

Blood and urine data are given for the first period of treatment. Table IV and Fig. 4 present the complete picture for the drug in the blood during the first two weeks of treatment. Rabbit 42 recovered more quickly than rabbit 41, although both maximum and minimum blood levels were lower in the former. The conjugation was strikingly different, uniformly low in rabbit 41, scarcely rising above 5 per cent of the total at any time, and uniformly much higher in rabbit 42. Conversely, the amount of free drug in the blood in rabbit 41 were uniformly high and relatively much greater than in rabbit 42. Whether this relatively higher level of free drug in rabbit 41 had any bearing on its slower recovery and manifestation of symptoms of toxic poisoning, including a temporary anemia, is uncertain. At any rate, this should be borne in mind along with the contention that free sulfanilamide is therapeutically more effective and that the conjugated drug is more toxic.

Elimination data are strikingly similar. There was an initial lag in recovery of the intake, then a rise up to a fairly even rate. During seventy-two hours of elimination subsequent to complete cessation of intake, almost identical amounts were recovered in the urine. This appears to represent the approximate amount of drug retained in the body during continuous treatment at the given dosage of the drug. Finally, a higher percentage of conjugated

TABLE IV

SULFANILAMIDE IN BLOOD AND URINE OF RABBITS: STREPTOCOCCUS INFECTION
(Intake of drug 1.0 Gm. per day = approximately 0.5 Gm. per kg. of body weight.)

DAYS TREATED	TIME OF BLOOD SPECIMEN	RABBIT 41 (RECOVERED) SULFANILAMIDE IN BLOOD: TOTAL MG. PER 100 ML.	RABBIT 41 (RECOVERED) SULFANILAMIDE IN BLOOD: PER CENT CONJUGATED	RABBIT 41 (RECOVERED) SULFANILAMIDE IN URINE: COLLECTION PERIOD HOURS	RABBIT 41 (RECOVERED) SULFANILAMIDE IN URINE: RECOVERY OF INTAKE PER CENT	RABBIT 41 (RECOVERED) SULFANILAMIDE IN URINE: PER CENT CONJUGATED	RABBIT 42 (RECOVERED) SULFANILAMIDE IN BLOOD: TOTAL MG. PER 100 ML.	RABBIT 42 (RECOVERED) SULFANILAMIDE IN BLOOD: PER CENT CONJUGATED	RABBIT 42 (RECOVERED) SULFANILAMIDE IN URINE: COLLECTION PERIOD HOURS	RABBIT 42 (RECOVERED) SULFANILAMIDE IN URINE: RECOVERY OF INTAKE PER CENT	RABBIT 42 (RECOVERED) SULFANILAMIDE IN URINE: PER CENT CONJUGATED
1	A.M.*										
	P.M.	16.95	1.1	24	48.5	17.5	16.4	19.8	24	47.1	53.6
2	A.M.	5.20	–				1.04	44.3			
	P.M.	18.8	4.3	24	47.5	21.5	15.15	25.4	24	76.7	58.6
3	A.M.	3.28	1.8				0.97	100.0			
	P.M.	17.4	1.7	24	74.3	15.9	15.6	26.6	24	69.9	60.4
4	A.M.	6.67	6.3				1.13	33.6			
	P.M.	17.3	0.0	24	62.3	19.1	16.5	34.2	24	56.5	57.0
5	A.M.	5.21	5.6				0.95	100.0			
	P.M.	16.95	0.0	24	59.2	16.3	15.56	30.6	24	59.3	56.8
6	A.M.	9.25	3.4				1.0	40.0			
7 & 8	No treatment on 6th, 7th, and 8th days			72	84.6	20.7	No treatment on 6th, 7th, and 8th days		72	83.5	58.4
9	A.M.	–	–				–	–			
	P.M.	15.6	3.9	24	55.1	19.0	17.23	48.2	24	44.8	58.0

*A.M. = morning specimen, eighteen to twenty hours after last dose (minimum blood level).
P.M. = evening specimen, two and one-half hours after last dose (maximum blood level).

TABLE V

SULFANILAMIDE IN BLOOD AND URINE OF RABBITS: STREPTOCOCCUS INFECTION
(Intake of drug 2.0 Gm. per day = approximately 1.0 Gm. per kg. of body weight.)

DAYS TREATED	TIME OF BLOOD SPECIMEN	RABBIT 10 (DIED, DRUG DEATH?) SULFANILAMIDE IN BLOOD: TOTAL MG. PER 100 ML.	RABBIT 10 (DIED, DRUG DEATH?) SULFANILAMIDE IN BLOOD: PER CENT CONJUGATED	RABBIT 10 (DIED, DRUG DEATH?) SULFANILAMIDE IN URINE: COLLECTION PERIOD HOURS	RABBIT 10 (DIED, DRUG DEATH?) SULFANILAMIDE IN URINE: RECOVERY OF INTAKE PER CENT	RABBIT 10 (DIED, DRUG DEATH?) SULFANILAMIDE IN URINE: PER CENT CONJUGATED	RABBIT 30 (DIED, DRUG DEATH?) SULFANILAMIDE IN BLOOD: TOTAL MG. PER 100 ML.	RABBIT 30 (DIED, DRUG DEATH?) SULFANILAMIDE IN BLOOD: PER CENT CONJUGATED	RABBIT 30 (DIED, DRUG DEATH?) SULFANILAMIDE IN URINE: COLLECTION PERIOD HOURS	RABBIT 30 (DIED, DRUG DEATH?) SULFANILAMIDE IN URINE: RECOVERY OF INTAKE PER CENT	RABBIT 30 (DIED, DRUG DEATH?) SULFANILAMIDE IN URINE: PER CENT CONJUGATED
1	A.M.*	–	–	–	–	–	–	–	–	–	–
	P.M.	–	–	–	–	–	22.5	30.0	24	24.4	35.6
2	A.M.	3.72	100.0				5.04	83.8			
	P.M.	19.11	27.8	24	38.8	64.1	31.2	36.5	24	1.1	51.0
3	A.M.	5.71	77.2				9.4	84.0			
	P.M.	26.15	33.4	24	19.0	62.0	30.3	37.2	24	0.9	68.0
4	A.M.	22.0	95.1				16.8	86.6			
	P.M.	24.6	56.5	24	15.8	61.2	48.6	27.1			
5	A.M.	13.5	69.2				No treatment				
	P.M.	37.9	–	24	30.8	72.7	on 5th day		48	93.2	52.0
6	A.M.	33.7	76.9								
		No treatment after 5th day; died two days later					Died two days later apparently from toxic effect of the drug				

*A.M. = specimen eighteen to twenty hours after last dose in case of both rabbits.
P.M. = specimen four and one-half hours after last dose for rabbit 10 and two and one-half hours after last dose for rabbit 30.

drug in the urine than in the blood reflects the greater ease of the elimination of the conjugated form, as previously indicated.

Treatment With 2 Gm. of Sulfanilamide Per Day.—Four series of rabbits were given this treatment. In series IV, the first of this group, both controls recovered. One of the treated animals, rabbit 10, died after five days of treatment. Death was apparently due to the effect of the drug, which was poorly eliminated and accumulated in the blood (Table V), gradually rising to a level of 33.7 mg. per cent. This appears to indicate an injury to the eliminating mechanism, possibly the kidneys. It should be noted that the amount of conjugated drug in the blood was continuously high after the second day of treatment, rising to above 25 mg. per cent. Conjugation, on the other hand, seems to be in accord with the usual finding in rabbits, indicating normal functioning of the liver at least so far as this conjugating mechanism is involved. The other treated rabbit showed a tendency to accumulation of drug in the blood. No urine studies were made. Death was due to an accident during treatment.

In series V, the second of this group, both controls died three weeks after inoculation. Hemolytic streptococci were cultured from the local lesions at autopsy. One of the treated animals died after one day of treatment. The infection was extremely acute, as evidenced by a temperature of 106° F. Hemolytic streptococci were cultured from the local lesion and from the peritoneal exudate. The other animal recovered after intermittent treatment over a period of five weeks. The characteristic picture in this animal consisted of flare-ups of infection after periodic discontinuance of the drug. This phenomenon was observed in other series. Recovery from the infection was complete at the end of the experiment. The drug was well handled, as judged by blood levels and elimination in the urine. Toward the end of the experiment a tendency toward accumulation of the drug in the blood was noted, along with lowered conjugation, which suggests injury to the mechanism of elimination and conjugating power of the liver. The maximum blood levels ranged around 20 to 25 mg. per cent. It may be that the high level during the last period of treatment, about 30 mg. per cent for the maximum values, was responsible for the final clearing up of the infection.

In series VI, the third of this group, one of the controls died of the infection. The other control recovered. In one of the animals, rabbit 24, treatment was continued over five weeks due to recurrent flare-ups of the infection. The drug was handled well during the first periods of treatment. Elimination and conjugation, as judged by blood and urine values, were apparently normal at first, but toward the end of the experiment there was, as in rabbit 25, a tendency to accumulation of drug in the blood and a definitely lowered tendency to conjugation. The maximum blood level values ranged around 20 mg. per cent during the first several periods of treatment, but thereafter, due to the accumulation in the blood, they rose at times to well above 30 mg. per cent. The temperature was subnormal for five days prior to death, and at autopsy no organisms could be demonstrated in the blood or from the site of injection. Death was undoubtedly caused by the drug.

The other animal died after treatment over a period of ten days. There was an accumulation of the drug in the blood which was manifest from the beginning. The amount of the conjugated form was high throughout, rising to a maximum of 33.8 mg. per cent. Elimination was normal at first, but decreased during the last two days. Although hemolytic streptococci were demonstrated in the local lesion at autopsy, we feel that death was primarily due to the drug.

In the final series of this group both controls died, one of them on the second day after inoculation. Autopsy revealed multiple coccidial abscesses in the liver, as well as hemolytic streptococci in the local lesion. The other control died of streptococcic infection in four days. One of the treated animals, rabbit 29, recovered (Fig. 3), but treatment had to be continued over two weeks, due to two flare-ups of infection. Maximum blood levels were high, repeatedly well above 40 mg. per cent. Conjugation was consistently low, so that the amount of conjugated drug in the blood rarely exceeded 10 mg. per cent, notwithstanding the high values for total drug. Elimination was low, less than half of the intake, which probably accounts for the high blood values. It is of particular interest to contrast the blood levels of rabbits 29 and 37, high in one and low in the other (Fig. 3). They responded to treatment in about the same manner. Both had to be treated repeatedly after recurrent flare-ups of infection. The amount of conjugated drug was consistently low over the entire period of treatment and neither manifested any toxic effects.

The other treated animal, rabbit 30, probably died from the effect of the drug. The blood level rose gradually to almost 50 mg. per cent. Elimination was poor and for two days there was an almost complete anuria.

Thus, in this group (series IV to VII), 5 of the controls died of the infection. Of the treated animals, 3 recovered from the infection after repeated flare-ups, but one of these died from the toxic effects of the drug. All of them manifested a definite tendency to drug retention toward the end of the treatment. The death of the other 4 treated animals may be ascribed to the effect of the drug, associated with an accumulation of the drug in the blood, a lowered elimination, and a definite anuria in at least one of these.

SUMMARY AND CONCLUSIONS

1. Blood and urine studies were made on the levels and distribution of sulfanilamide in seven series of rabbits infected with hemolytic streptococci.

2. All the animals received similar inoculations, but marked individual differences of susceptibility to the infective organism were noted.

3. Recurrent flare-ups of infection were observed in a number of animals. Complete clinical cure required resumption of treatment at varying intervals.

4. A definite tendency toward accumulation of the drug in the blood and poor elimination was noted in the majority of the treated animals and was a contributory cause of death in at least 5. In all the animals in which the drug was a definite factor in causing death, the level of the conjugated form was relatively high.

5. Most favorable results in the treatment of infection were obtained with the highest dosage used, approximately 1 Gm. per kilogram of body weight, with a drug level ranging between 20 and 30 mg. per cent.

6. For a given intake of the drug the elimination in the urine reaches a maximum level within two or three days after the beginning of treatment. The conjugated drug is more readily eliminated than free sulfanilamide, irrespective of the level in the blood.

We wish to express our appreciation to Professors Randle C. Rosenberger and George R. Bancroft whose interest has made it possible to carry out this investigation.

REFERENCES

1. Marshall, E. K., Jr.: Determination of Sulfanilamide in Blood and Urine, J. Biol. Chem. **122:** 263, 1937.
2. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C.: Para-Aminobenzene-sulfonamide. Absorption and Excretion; Method of Determination in Urine and Blood, J. A. M. A. **108:** 953, 1937.
3. Hansen, L.: Sulfanilamide Concentration and Distribution in Blood and Urine in Sulfanilamide Therapy for Gonococcus Infections in Men, J. Pharmacol. & Exper. Therap. **65:** 372, 1939.
4. Marshall, E. K., Jr., Cutting, W. C., and Emerson, K., Jr.: The Toxicity of Sulfanilamide, J. A. M. A. **110:** 252, 1938.
5. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C.: The Distribution of Sulfanilamide in the Organism, J. Pharmacol. & Exper. Therap. **61:** 196, 1937.
6. Lucas, C. C.: The Human Response to Single Doses of Sulphanilamide, Canad. M. A. J. **39:** 111, 1938.
7. Feinstone, W. H., Bliss, E. A., Ott, Earl, and Long, P. H.: Observations Concerning the Toxicity, Absorption and Therapeutic Effect of Sulphanilamide and Certain Related Organic Sulphur-Containing Compounds in Experimental Infections in Mice, Bull. Johns Hopkins Hosp. **62:** 565, 1938.
8. Marshall, E. K., Jr., and Cutting, W. C.: Absorption and Excretion of Sulfanilamide in the Mouse and Rat, Bull. Johns Hopkins Hosp. **63:** 328, 1938.
9. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C.: The Renal Excretion of Sulfanilamide, J. Pharmacol. & Exper. Therap. **61:** 191, 1937.

THE CALORIGENIC ACTION OF AMMONIUM SALTS IN THE HUMAN SUBJECT*

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Grafe[1] reported his studies of the specific dynamic action of protein in rabbits, dogs, and men. He considered ammonium carbonate, ammonium chloride, acetamide, and asparagine per gram of nitrogen comparable to glycine and alanine in increasing heat production. He concluded that the nitrogen of amino acids was the principal factor in their specific dynamic action and the combustile fraction was secondary. Of the substances he tested, ammonium chloride had a much greater calorigenic effect than the amino acids. Lundsgaard[2] compared the calorigenic action of sodium and ammonium glycocollate to that of glycine and that of sodium and ammonium lactate to that of alanine in normal conscious dogs. He reported that the sodium salts had slight effect, while that of the ammonium salts was very similar to that of the corresponding amino acids. This report is an extension of Lundsgaard's studies in the human subject.

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TABLE I

EFFECT OF AMINO ACIDS AND AMMONIUM AND SODIUM SALTS OF THE CORRESPONDING ACIDS UPON OXYGEN CONSUMPTION

SUBJECT	TEST SUBSTANCE	OXYGEN CONSUMPTION						NUMBER OF TRIALS
		BASAL C.C. PER MIN.	PER CENT INCREASE ABOVE BASAL				AVERAGE OF MAXIMUM INCREASE OF ALL TRIALS	
			MINUTES AFTER INGESTION OF DRUG					
			30	60	90	120		
C	Alanine	220	12.6	15.7	5.2	2.1	17.8	2
	Alanine	200	15.5	20.2	-	15.5		
	Ammonium lactate	209	3.1	11.1	20.0	11.1	20.5	5
	Ammonium lactate	209	13.0	21.7	13.0	0		
	Sodium lactate	208	8.4	8.4	3.0	-	10.1	6
	Sodium lactate	218	1.2	1.2	6.3	7.6		
	Repeated basals	202	2.8	0	0	5.6		3
N	Alanine	214	25.3	-	34.0	9.1	22.5	2
	Alanine	212	11.1	11.1	8.4	8.4		
	Ammonium lactate	220	8.2	-	13.4	5.6	13.6	5
	Ammonium lactate	220	7.5	18.0	18.0	13.0		
	Sodium lactate	209	5.7	5.7	5.7	8.4	6.7	5
	Sodium lactate	212	9.0	3.1	5.8	3.1		
	Repeated basals	213	0	0	0	5.7		3
J	Alanine	236	1.2	5.2	0	1.2	6.5	
	Alanine	228	-	11.6	7.4	7.4		
	Ammonium lactate	237	17.1	16.0	19.9	9.7	18.2	
	Ammonium lactate	236	20.9	13.8	11.0	13.8		
	Sodium lactate	228	0	5.7	12.2	9.6	11.1	
	Sodium lactate	244	9.5	6.8	4.5	0		
	Repeated basals	229	0	2.5	2.5	0		3
R	Glycine	216	8.7	12.0	10.1	-	12.6	2
	Glycine	203	13.3	13.3	12.3	-		
	Ammonium acetate	212	11.2	11.2	11.2	12.5	7.9	4
	Ammonium acetate	211	7.5	7.5	-	-		
	Sodium acetate	221	8.5	4.5	-	-	5.0	4
	Sodium acetate	233	0	0	-4.2			
S	Glycine	222	8.1	8.1	8.1		10.9	2
	Glycine	211	4.7	13.7	9.0			
	Ammonium acetate	226	16.3	-	-		14.6	3
	Ammonium acetate	241	2.1	17.4	9.9			
	Sodium acetate	213	8.9	8.9	12.2		11.4	2
	Sodium acetate	227	0	4.5	-			
Mc	Glycine	195	8.9	3.5	3.5		6.0	2
	Ammonium acetate	200	9.9	19.8	23.4		16.3	5
	Ammonium acetate	181	16.0	19.9	8.8			
	Sodium acetate	196	5.1	5.9	0			
	Sodium acetate	197	0	0	5.1		5.4	3

EXPERIMENTAL PROCEDURE

The oxygen consumption of 6 normal medical students was measured by a Benedict-Sanborn metabolism machine. The subjects were accustomed to breathing through the apparatus before data collection began. The tests were made in the morning at least fifteen hours after the last meal and after a thirty-minute rest upon a cot. The basal oxygen consumption was ascertained, and the test substance was administered orally in 75 c.c. of water in a dosage of 0.133 molar. The oxygen consumption was then measured thirty, sixty, ninety, and one hundred and twenty minutes after ingestion of the test substance. The alanine was a racemic mixture. The basal oxygen values were corrected for temperature and barometric pressure.

Control observations were made by the ingestion of 75 c.c. of water, an isomolar amount of sodium bicarbonate or sodium chloride, or repeated basals without the ingestion of any substance. The psychic reactions to the administration of these substances were very slight.

RESULTS

To conserve space only representative data are presented in Table I, together with the average maximum increase for each substance. The maximum variation in the controls with water and repeated basals never exceeded 4 per cent. The increases effected by the sodium salts, lactate, acetate, bicarbonate, and chloride were about the same, ranging from 5.7 to 10.7 per cent. On the other hand, the average calorigenic effect of ammonium lactate was 89 per cent greater than that of sodium lactate and that of ammonium acetate was 138 per cent greater than that of sodium acetate. The ammonium salts were more effective than the corresponding amino acids. For the most part, the response to the effective drugs came at sixty to ninety minutes, and to the sodium salts at thirty minutes. The length of the latent period rules out any psychic effects of taking the drugs. The duration of the response was greatest for alanine, and the ammonium salts, being 120 minutes or more. For the sodium salts, the effect seldom lasted longer than eighty minutes. The urine output in the two hours after taking the salts was always less than after the amino acids.

DISCUSSION

The data indicate that ammonia exerts a definite calorigenic effect in the human body. It might well be considered as additional evidence supporting the idea advanced by others[3] that the ammonia arising from the deaminization of amino acids is largely responsible for the specific dynamic action of protein. In our experiments the sodium salts of acetic and lactic acids had no greater effect than the bicarbonate or chloride. This might indicate that the residue after deaminization has no appreciable specific calorigenic action and that the effect of all the sodium salts in these experiments could be ascribed to osmotic pressure changes. The shorter latent period for maximum response to the sodium salts than for the ammonium salts supports this idea.

The mechanism by which ammonia exerts a calorigenic action is unknown, but it has been demonstrated to exert effects upon the metabolism of tissues in vitro. Annau[4] and Edson[5] reported that ammonia increased the rate of acetoacetic acid formation in liver slices. Edson found that ammonium chloride increased the respiration of kidney tissue, had no effect upon brain or spleen, and slightly inhibited the striated muscle. Krebs[6] has reported that in the presence of glutamic acid, kidney and nervous tissue take up large quantities of ammonia to form glutamine. In the brain and retina the quantity of ammonia disappearing exceeded the amount of amide nitrogen formed. Neber[7] has shown that surviving rat liver forms large quantities of amino nitrogen when ammonium pyruvate is added. Since the formation of the amino acids proceeds in the absence of oxygen, the reaction must be exothermic. Krebs and Henseleit[8] reported that urea formation from ammonia in liver slices re-

quired tissue respiration. Borsook and Jeffreys,[9] after theoretical considerations, decided that urea formation from ammonia could not occur spontaneously but must depend upon an energy-yielding reaction. Some of these reactions or effects might have occurred in our experiments and caused either directly or indirectly increased heat production.

The responses of subject J to the ingestion of alanine were only 2.4 and 5.2 per cent increase, but when the amount of alanine was doubled, the per cent increase was 11.8. About six months after these experiments, the subject's physical complaints led to the diagnosis of a moderate degree of diabetes mellitus. In this connection it is interesting to note that Grafe found that the administration of 50 Gm. of glycine to a depancreatized dog resulted in a 10 per cent decrease in oxygen consumption and carbon dioxide production.

SUMMARY

In 3 normal subjects, with a dosage of 0.133 molar, the ingestion of alanine produced an average increase in oxygen consumption above the basal value of 14.6 per cent; ammonium lactate 17.4 per cent and sodium lactate 9.2 per cent. In 3 other human subjects the ingestion of glycine produced an average increase in oxygen consumption above the basal level of 9.8 per cent, ammonium acetate 13.6 per cent, and sodium acetate 5.7 per cent. Isomolar doses of sodium bicarbonate and sodium chloride caused oxygen consumption to be increased on the average of 10.7 and 9.9, respectively.

The comparable calorigenic effects of these ammonium salts to the corresponding amino acids, alanine, and glycine are in agreement with the studies of Grafe and Lundsgaard. The marked difference in the calorigenic effects of the ammonium and sodium salts might be interpreted to mean that the specific dynamic action of amino acids is due to their amino group and not to their non-nitrogenous fraction. The similarity of the calorigenic effects of the sodium salts of either organic or inorganic acids, and the shorter latent period for maximum response, might be attributed to osmotic effects rather than to oxidative processes.

REFERENCES

1. Grafe, E.: Beiträge zur Henntnis der Ursachen der spezifischdynamischen Wirkung der Eiweisskörper, Deutsches Arch. f. klin. Med. **118**: 1, 1916.
2. Lundsgaard, Einar: Über die Ursachen der Spezifischen dynamischen Wirkung der Nahrungsstoffe, Skandinav. Arch. f. Physiol. **62**: 243, 1931.
3. Wilhelmji, Charles M.: The Specific Dynamic Action of Food, Physiol. Rev. **15**: 202, 1935.
4. Annau, Ernö: Über den chemischer Verlauf und die physiologische Bedingungen der Acetonkörferbildung aus Brenztraubensaure, Ztschr. f. physiol. Chem. **224**: 141, 1934.
5. Edson, Norman Lowther: The Influence of Ammonium Chloride on Ketone-Body Formation in Liver, Biochem. J. **29**: 2082, 1935.
6. Krebs, Hans Adolf: The Synthesis of Glutamine From Glutamic Acid and Ammonia, and the Enzymic Hydrolysis of Glutamine in Animal Tissues, Biochem. J. **26**: 1951, 1935.
7. Neber, M.: Über die Aminosäuresynthese aus Ketosäure und die Harnstoffsynthese in der Leber, Ztschr. f. physiol. Chem. **234**: 83, 1935.
8. Krebs, Hans Adolf, and Henseleit, Kurt: Untersuchungen über die Harnstoffbildung im Tierkörper, Klin. Wchnschr. **11**: 1137, 1932.
9. Borsook, Henry, and Jeffreys, Cecil E. P.: Nitrogen Metabolism of the Isolated Tissues of the Rat, J. Biol. Chem. **110**: 495, 1935.

THE TOXIC EFFECTS OF SULFANILAMIDE UPON TISSUES OF RATS*

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THE introduction into therapeutics of prontosil I by Domagk[1] in 1935 was followed by widespread clinical and experimental investigations which have confirmed Domagk's original observations. In 1935 Tréfouël, Nitti, and Bovet[2] came to the conclusion that it was the sulfonamide grouping which was responsible for the therapeutic activity of prontosil I. Their work was confirmed in 1936 by Goissedet, Despois, Gailliot, and Mayer,[3] Buttle, Gray, and Stephenson,[4] Gley and Girard,[5] and Colebrook and his co-workers.[6] The reduction of prontosil I and II was accomplished by Bliss and Long[7] in 1937. Shortly thereafter, it was demonstrated (Fuller[8]) that the oral or parenteral administration of prontosil I and II or of sulfanilamide was followed by the appearance of free sulfanilamide and acetyl sulfanilamide in equal proportions in blood and urine. Considerable success has attended the use of these compounds in streptococcal, meningococcal, and gonococcal infections in human beings. Much speculation has arisen regarding the mechanism of the therapeutic activity and, in Table I, is presented a brief summary of the hypotheses which have been put forward by various workers.

Table I

Views Concerning Mode of Action of Sulfanilamide

Action upon microorganism	Action upon host
1. Bacteriostasis (Colebrook et al.; Bliss and Long)	1. Activation of phagocytic activity of cells (Domagk)
2. Inhibition of formation of hemolysins and negatively chemotactic substances (Levaditi and Vaisman; Meyer; Osgood and Brownlee)	2. Stimulation of reticulo-endothelial system (Burgers; Gley and Girard; Levaditi and Vaisman)
3. Interference with capsule formation or capsule destruction (Levaditi; Lyons)	3. Combination of sulfanilamide with body protein rendering it unfit medium for microorganisms (Levaditi)
4. Interference with protein-digesting enzymes (Lockwood)	
5. Bactericidal (Frankl)	

It has become apparent from widely scattered experimental and clinical reports that sulfanilamide and related compounds possess a definite toxicity both for man and for the lower animals. The toxic dosage in different species

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of animals, and even in different individuals of the same species, varies. The present investigation has been undertaken to determine the pathologic changes in the tissues of rats following prolonged and excessive administration of sulfanilamide.

TOXICITY OF SULFANILAMIDE

Much uncertainty still exists regarding the maximum tolerated dose and the minimum lethal dose of sulfanilamide in various animals. The tolerated amount in mice has been placed at 2.5 Gm. per kilogram (Buttle, Gray, and Stephenson[4]), 0.9 Gm. per kilogram (Long and Bliss[9]), 2 Gm. per kilogram (Raiziss, Severac, and Moetsch[10]), 2.5 Gm. per kilogram (Whitby[11]), and 3 to 4 Gm. per kilogram (Rosenthal[12]). In rabbits the maximum tolerated dose has been found to be 1 Gm. per kilogram (Tréfouël and co-workers[2]), 5 Gm. per kilogram (Nitti and Bovet[14]). The fatal dose in mice varies from 3 Gm. per kilogram (Raiziss, Severac, and Moetsch[10]) to 7.5 Gm. per kilogram (Whitby[11]), and 10 Gm. per kilogram (Buttle, Gray, and Stephenson[4]).

TABLE II

SUMMARY OF TOXIC EFFECTS OF SULFANILAMIDE

HUMAN BEINGS	LOWER ANIMALS
1. Nausea	1. Spasticity of extremities
2. Vomiting	2. Excitability
3. Dizziness	3. Vestibular dysfunction
4. Malaise	4. Ataxia
5. Headache	5. Diminished reflex, excitability
6. Dermatitis	6. Motor paralysis
1. Angioneurotic edema	7. Partial narcosis
2. Maculopapular	8. Hypothermia
3. Morbilliform	9. Cardiac depression
7. Fever	10. Fall of blood pressure
8. Acidosis	11. Loss of weight
9. Leucocytosis	
10. Hemolytic anemia	
11. Agranulocytosis	
12. Methemoglobinemia	
13. Sulfhemoglobinemia	
14. Jaundice with decrease in liver function	
15. Acute yellow atrophy of liver	
16. Peripheral and optic neuritis	
17. Nephritis	

The toxic symptoms in these animals varied (Table II): spasticity of the extremities, excitability, incoordination, vestibular dysfunction, ataxia, somnolence, lessened reflex excitability, motor paralysis, deepened respiration, and partial narcosis. A reduction of the body temperature and of the respiratory quotient has been noted in rats. Large intravenous doses produce a fall in blood pressure which is associated with a direct depressant action upon the cardiac muscle and a dilatation of the visceral blood vessels. These toxic effects are of an acute nature and do not resemble the symptoms of toxicity seen in human beings.

Various toxic manifestations have been noted in human beings in response to the administration of sulfanilamide (Table II). In a series of 335 patients Long, Bliss, and Feinstone[15] observed dizziness, headache, loss of ability to

concentrate, nausea, and vomiting in many. A considerable number of these patients demonstrated the presence of cyanosis with or without methemoglobinemia and sulfhemoglobinemia. Acidosis associated with hyperpnea and a lowered carbon dioxide combining power of the blood occurred in 3 per cent of the patients. Jaundice accompanied by a diminution of liver function was present in one. Skin rashes were noted in 1 per cent of the patients and fever in 6 per cent. They found that a chronic hemolytic anemia developed in a considerable number, while acute hemolytic anemia was seen in 3 per cent of their series. Agranulocytosis was found in one patient. Fatal agranulocytosis has been reported by several observers (Young,[16] Plumer,[17] Berg and Holtzman,[18] O'Connell,[19] Schwartz and co-workers,[20] Shecket and Price,[21] and others). Death from acute yellow atrophy of the liver, as a result of the administration of sulfanilamide, has been reported (Cline[22]). Peripheral neuritis (Ornsteen and Furst[23]) and optic neuritis (Buey[24]) have been included among the toxic manifestations.

METHODS

A series of 80 white rats, each weighing from 200 to 250 Gm., were used in this investigation. Of these, 40 served as controls and the remainder were divided into 4 groups of 10 rats to each group. The animals were observed for a period of two weeks before being used, and all unhealthy ones were discarded. An adequate amount of water and food was provided. Sulfanilamide,* in a 10 per cent suspension in sterile doubly distilled water, was injected beneath the skin of the anterior abdominal wall daily over a period of three months. All equipment used was sterilized before each injection, and every effort was made to maintain a reasonable degree of sterility. The following dosages of sulfanilamide were used: B group received 0.5 Gm. per kilogram of body weight; C group received 1.0 Gm. per kilogram; D group received 1.5 Gm. per kilogram; E group received 2.0 Gm. per kilogram. At intervals of ten days from the commencement of the experiment, one animal from each group was killed by a blow upon the head. The tissues were immediately removed and placed in a 10 per cent formalin solution, or in absolute alcohol (where glycogen stains were to be made). The tissues were embedded in celloidin, and the following stains were used: hematoxylin-eosin, Best's carmine stain for glycogen, Mallory's stain for iron, scarlet red stain for fat.

RESULTS

Shortly after the injections were made the animals exhibited a marked disinclination to movement and remained stationary in one corner of the cage. Some of the rats, however, appeared to be unaffected by the sulfanilamide and remained active. Many showed a considerable degree of thirst during the first few days of injection. No exact data were obtainable concerning the amount of water intake and output, but our observations indicated an increased water intake during the earliest periods of the administration of the drug. By the end of the first week, however, a normal level of water intake had been at-

*We are indebted to the Medical Research Division, Eli Lilly Co., Indianapolis, Ind., for kindly supplying us with the sulfanilamide used in this investigation.

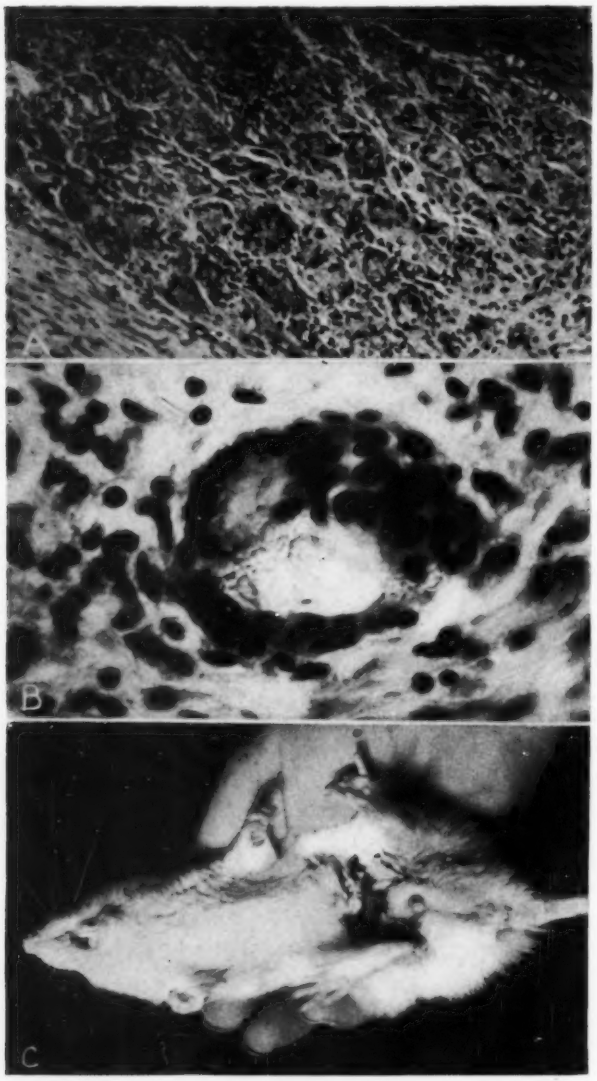

Fig. 1.—*A*, Subcutaneous tissue of anterior abdominal wall (×175). Foreign body reaction with giant cell formation. *B*, The same specimen (×625). Sulfanilamide crystal lying within giant cell. *C*, Rat: necrosis of skin of anterior abdominal wall.

tained. Some of the animals revealed a considerable degree of cyanosis of the mucous membranes of the mouth and tongue, which apparently was not related definitely to the amount of sulfanilamide given. It soon became apparent that the greatest reaction to the sulfanilamide was exhibited during the first few days of the injection and, if the animal could withstand the toxic action during this period, it was likely to survive indefinitely. It was found to be possible to increase the tolerance to the drug gradually, so that in some animals as much as 6 Gm. per kilogram were injected without harmful results ensuing. The mechanism of this induced tolerance is unknown. Another factor, which became evident, was the individual idiosyncrasy of these animals to

sulfanilamide. Some exhibited a reaction to the smallest amount given and others were apparently unaffected even by large amounts. Most of the animals showed a moderate weight loss which became accentuated when, and if, necrosis occurred at the site of injection.

Pathology at Injection Site.—The subcutaneous deposition of the amorphous crystals of sulfanilamide resulted in changes, both in the crystals and in the surrounding tissue. After a few days the crystals began to disintegrate into smaller needle-shaped particles and, finally, into a granular appearing mass in which all traces of a crystalline structure had disappeared. Complete disappearance of the injected material took place relatively slowly, and traces were still present as long as two months after the final injection. The first local response to the introduction of the sulfanilamide into the tissue was a marked infiltration by inflammatory cells, most of which were wandering mononuclear cells and lymphocytes. This was followed by proliferation of the local histiocytes and fibrous connective tissue cells. Numerous giant cells of the Langhans type formed, and in many of these could be seen crystalline and granular material. The proliferating fibroblasts showed the presence of frequent mitotic figures (Fig. 1, *A* and *B*). A moderate degree of tissue eosinophilia was also evident. This foreign body response in the tissues varied in each animal quantitatively but not qualitatively. It was most apparent during the earlier periods of injection of sulfanilamide. After a period of two months examination of the injection site revealed the presence of adult fibrous connective tissue showing few mitotic figures. A moderate number of mononuclear cells was scattered throughout the tissue. Giant cells were absent or few in number. Moreover, practically all the injected sulfanilamide crystals had been removed. Necrosis occurred at the site of injection in several animals and was usually associated with a moderate grade of infection (Fig. 1, *C*).

TABLE III

BLOOD PICTURE IN CONTROL RATS

GROUP	RED BLOOD CELLS	HEMOGLOBIN GM.	WHITE BLOOD CELLS
A-3	6,250,000	14.8	2,000
A-4	5,550,000	16.8	4,400
A-5	6,700,000	16.6	3,520
A-6	6,400,000	16.2	3,950
A-7	5,250,000	13.6	2,900
A-8	6,100,000	16.1	4,500
A-9	5,250,000	13.5	2,300
A-10	5,600,000	12.8	3,100
Average	5,887,500	15.03	3,333

Blood Picture.—Complete blood studies were made in many of the control series and in most of the sulfanilamized rats. Blood was obtained in both series of animals in an exactly similar manner, by killing the rats and taking blood directly from the heart. It will be noted (Table III) that representative data indicate that, in normal rats, the erythrocytes vary from 5,250,000 per cm. to 6,700,000 per cm., with an average count of 5,887,500 per cm. The hemoglobin varied from 12.8 Gm. to 16.8 Gm. per 100 c.c. of blood, with an average of 15.03 Gm. per cent. The leucocyte count varied from 2,000 per

cm. to 4,500 per cm., with an average count of 3,333. The sulfanilamized rats revealed a definite reduction of both the red blood cells and the hemoglobin. This reduction was directly proportional to the amount of sulfanilamide given (Table IV). However, the factor of individual idiosynerasy to the drug was present, but was evidently minor to that of dosage. In some of these animals the anemia was quite severe. The mechanism of this anemia must be subjected to examination. Two possibilities may be considered: (1) a direct action of sulfanilamide upon the erythropoietic elements in the bone marrow; (2) a direct hemolytic action of the drug upon the circulating erythrocytes. Evidence will be presented later in this paper which supports the view that this anemia is hemolytic in origin. Representative data (Table IV) regarding the leucocyte count reveal several interesting facts. While the average count indicates a mild leucocytosis, a further analysis discloses the fact that with B group of rats (0.5 Gm. per kilogram) the count is unaffected.

TABLE IV

INFLUENCE OF SULFANILAMIDE UPON BLOOD OF RATS

GROUP	RED BLOOD CELLS	HEMOGLOBIN GM.	WHITE BLOOD CELLS
B-1	4,500,000	13.8	3,200
B-2	3,700,000	13.2	3,520
C-2	5,200,000	13.2	5,200
C-4	5,350,000	14.8	8,000
D-2	4,800,000	12.0	5,400
D-3	3,700,000	10.2	3,100
D-4	4,950,000	13.1	6,400
D-5	5,350,000	15.0	6,400
E-2	2,300,000	11.0	3,400
E-5	3,900,000	12.0	3,100
Average	4,375,000	12.83	4,772

C group (1.0 Gm. per kilogram) shows a definite leucocytosis, as does also group D (1.5 Gm. per kilogram). However, in group E (2.0 Gm. per kilogram) the count has returned to normal or slightly below normal. Apparently, dosage is important, inasmuch as moderate amounts of sulfanilamide cause leucocytosis, while large amounts return the count to normal or lower. The tentative conclusion might be drawn that sulfanilamide affects the red blood cells more readily than the white blood cells and that the earliest evidence of toxicity is a leucocytosis and later a leucopenia.

Bone Marrow.—The bone marrow was obtained by sectioning the left femur at the junction of the middle and upper thirds. The changes found were slight in most animals. A mild hyperplasia of the myeloid elements of the bone marrow and an increase in the number of megakaryocytes (Fig. 2, *A*) occurred. No other significant alterations were present. However, in one rat of the sulfanilamized series the bone marrow revealed a definite arrest of maturation of the myeloid cells (Fig. 2, *B*). These cells were seen to consist mainly of myeloblasts and early myelocytes. No lobing of the nuclei could be detected, and there was a complete absence of mature polymorphonuclear leucocytes. This bone marrow also revealed a decrease in the number of megakaryocytes. The total cellularity of the marrow was not diminished.

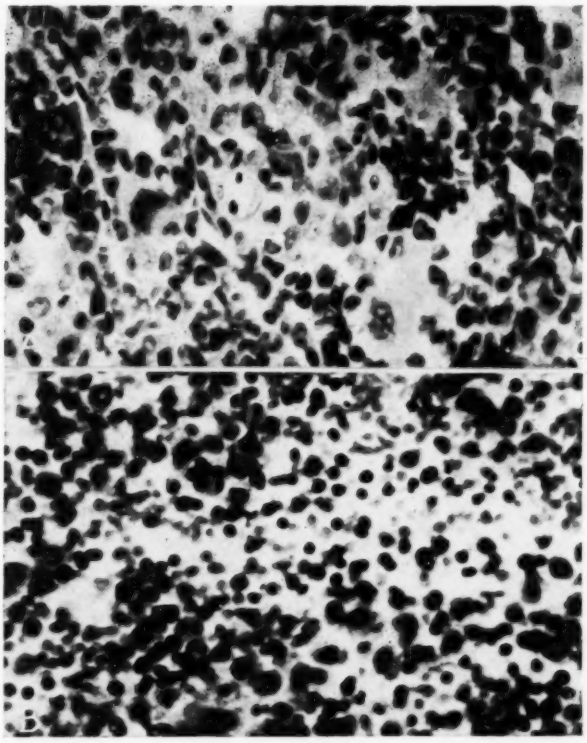

Fig. 2.—*A*, Femoral bone marrow (×625). Hyperplasia of myeloid elements. *B*, Femoral bone marrow (×625). Arrest of maturation of myeloid cells.

Spleen.—The spleen exhibited several interesting changes. In a number of animals, one or more nodular protuberances occurred upon the surface of the splenic capsule (Fig. 3, *A*). These consisted of a proliferation of young fibroblasts in which small amorphous crystals could be found. Occasionally, a foreign body giant cell, containing a crystal, was noted, as was also a mild infiltration by mononuclear cells and eosinophile leucocytes (Fig. 3, *B*). The mechanism of the crystal deposition will receive comment later. Examination of the splenic pulp revealed a hyperplasia of the reticulum and endothelial cells of varying degree. In only 3 animals was this marked; in these the hyperplastic cells appeared to be causing some compression of the blood spaces (Fig. 3, *C*). In several animals areas of focal necrosis were present and appeared to favor the centers of the Malpighian bodies. In such foci the cells were swollen or disintegrating with karyolysis and karyorrhexis of the nuclei and secondary invasion by polymorphonuclear leucocytes (Fig. 3, *D*). In the spleens of both the control rats and the sulfanilamized rats there was present a brownish pigment, lying in the form of granules within the reticulo-endothelial cells. However, greater amounts of this pigment were found in the spleens of the sulfanilamized animals. Appropriate stains indicated that this pigment contained iron and was probably derived from the breakdown of erythrocytes. The presence of anemia and the occurrence of iron-containing pigment in the spleen point to an excessive destruction of red blood cells. It is not possible to state, from data

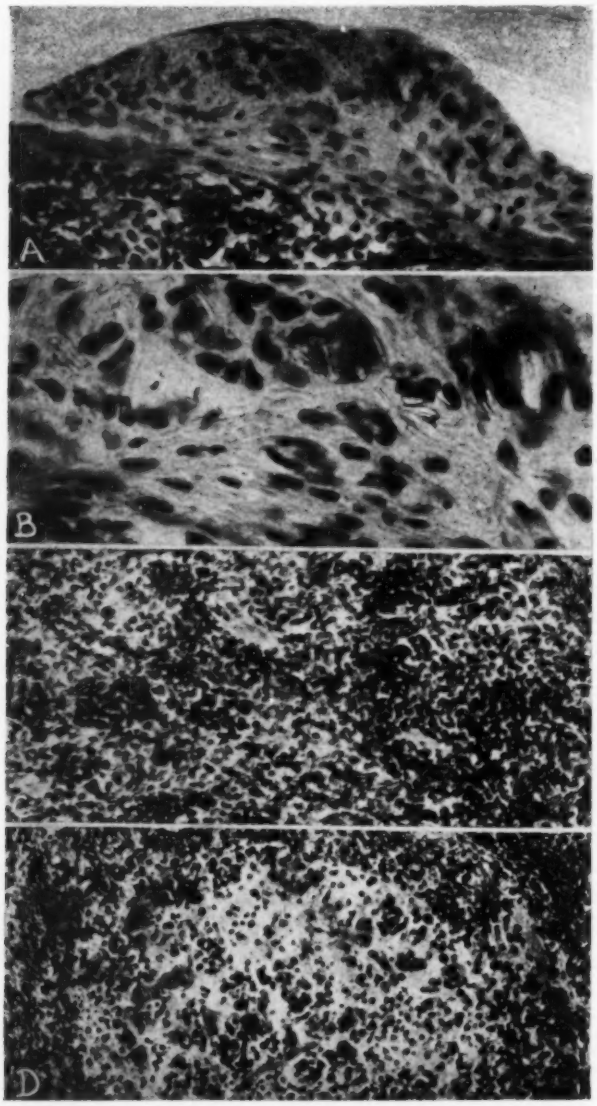

Fig. 3.—*A*, Capsule of spleen (×175). Fibroblastic nodule with crystal deposition. *B*, The same specimen (×625). *C*, Spleen (×175). Hyperplasia of reticulum and endothelial cells. *D*, Spleen (×175). Necrosis in Malpighian nodule.

obtained, whether or not this red blood cell destruction is due to a direct effect of sulfanilamide or to an excessive splenic activity in this direction. In one rat the spleen showed extramedullary hematopoiesis in the form of islands of myeloid cells. The significance of this finding is problematical.

Liver.—No significant changes in the size of the liver were found in the sulfanilamized animals. The liver cells revealed varying grades of alteration. The cells were enlarged, and the cytoplasm revealed the presence of numerous coarse granules (Fig. 4, *A*). In several animals mitoses in the liver cells were increased in number. A frequent finding was the formation of nodules of fibrous connective tissue on the outer and inner surfaces of Glisson's capsule.

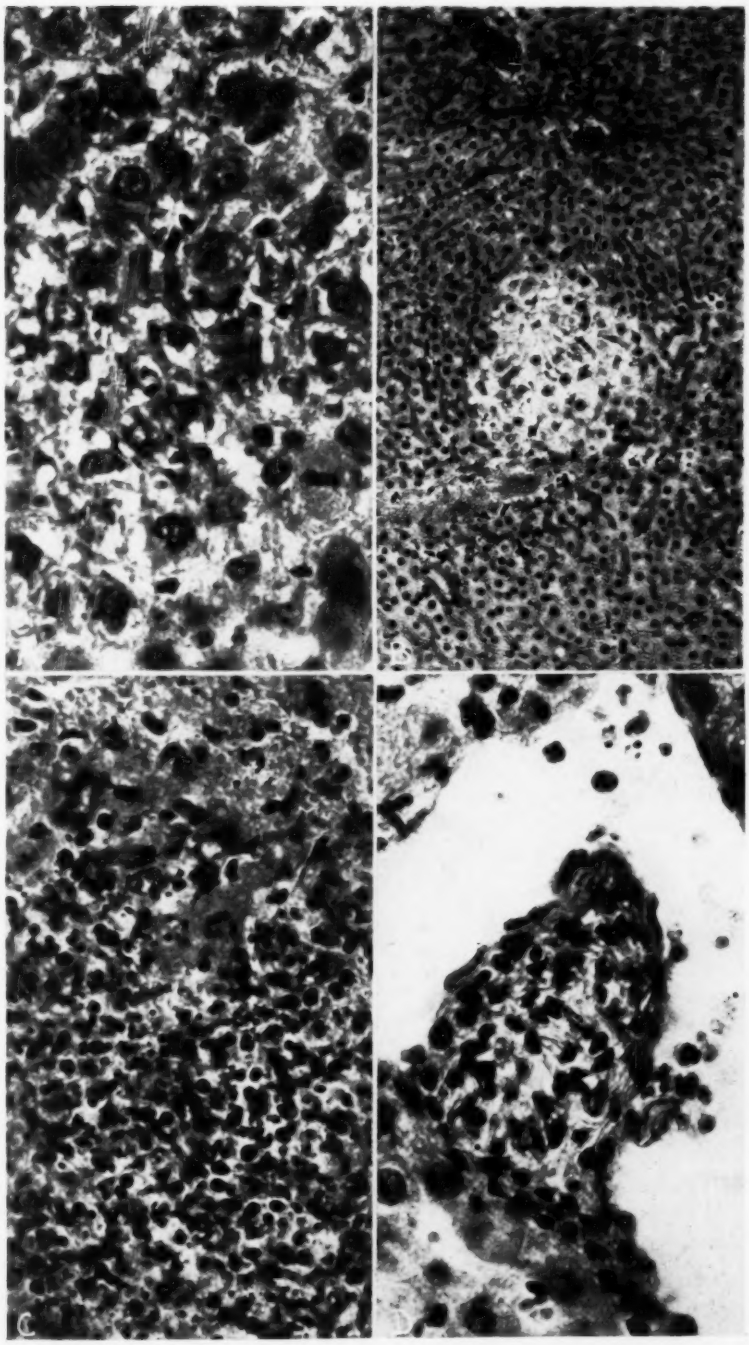

Fig. 4.—*A*, Liver (×625). Parenchymatous degeneration of hepatic cells; occasional mitoses. *B*, Liver (×175). Early focal necrosis. *C*, Liver (×625). Focal necrosis with cellular exudate. *D*, Liver (×625). Intimal nodule projecting into lumen of blood vessel; crystal deposition.

In these nodules crystal deposition could be noted. Similar formations were present within the liver and were perivascular in distribution. Occasional projections from the intima of the larger blood vessels of the liver were seen. These were composed of fibrous connective tissue in which crystals could be found (Fig. 4, *D*). In several instances irregularly placed focal areas of necrosis of the liver cells occurred. In the earliest stages the hepatic cells became pale and swollen (Fig. 4, *B*). Still later, an infiltration of polymorphonuclear leucocytes and wandering mononuclear cells took place into these areas (Fig. 4, *C*). Not infrequently, giant cells of the foreign body type could be seen surrounded by polymorphonuclear leucocytes and monocytes. No crystal deposition was noted in these focal areas of necrosis. The Kupffer cells of the liver were more prominent than usual, and many of them contained a brown pigment which was subsequently determined to be hemosiderin. Repeated fat stains were made of these livers, but only a slight increase in the amount of stainable fat was present. Glycogen stains did not reveal any abnormal changes.

TABLE V

INCIDENCE OF TISSUE ALTERATIONS IN SULFANILAMIZED RATS

TYPE OF TISSUE CHANGE	NUMBER OF RATS PRESENTING CHANGE	PER CENT
1. Foreign body reaction at injection site	40	100
2. Secondary anemia	30	75
3. Myeloid hyperplasia in bone marrow	15	37.5
4. Myeloid arrest in bone marrow	1	2.5
5. Hemosiderosis of spleen and liver	30	75
6. Parenchymatous degeneration of liver cells	5	12.5
7. Focal necrosis of liver	4	10
8. Focal necrosis of spleen	1	2.5
9. Crystal deposition in liver, spleen, and meninges	10	25
10. Hyperplasia of reticulo-endothelial cells of spleen	6	15
11. Myelopoiesis in spleen	1	2.5

Kidney.—Examination of the kidneys revealed only minor alterations. A moderate degree of cloudy swelling of the epithelium of tubules, particularly that of the convoluted tubules, was noted. The glomeruli showed no significant pathology. A mild increase of fat could be detected in the tubular epithelium by the use of appropriate stains.

Nervous System.—In several animals there occurred microscopic proliferations of fibrous connective tissue in the meninges. In these areas deposits of small crystals, of the same type described elsewhere, were present. No other significant changes were present in the brain, spinal cord, or peripheral nerves.

Lungs.—A mild increase in the number of macrophage cells was noted in the sulfanilamized animals. Several rats exhibited bronchopneumonia, but this was probably an incidental finding.

Heart.—No significant alterations were found.

Adrenal Gland.—No pathology was noted.

COMMENT

It is evident from this study that the toxic properties of sulfanilamide manifest themselves not only in functional disturbances but also in definite

alterations of the tissues. An examination of the literature reveals a conflict of opinion regarding the tissue pathology following administration of sulfanilamide. An increased amount of fat has been noted in the epithelium of the collecting tubules of the kidneys of rats, rabbits, and dogs following sulfanilamide, but no other tissue lesions (Geiling and Cannon[25]). Hawking[26] found no lesions in the kidneys or other viscera, but reported chromatolytic changes in the neurones of the spinal cord, cerebral cortex, and midbrain. Using mice, Hageman[27] injected sulfanilamide both subcutaneously and intraperitoneally in a dosage of 1 to 2.5 Gm. per kilogram. He reported no evidences of visceral pathology, but noted splenic hemosiderosis and an eosinophilia in the bone marrow. The intraperitoneal injections produced a foreign body reaction in the peritoneum, with crystals of sulfanilamide scattered throughout the area of reaction. Cloudy swelling of the tubular epithelium of the kidneys and of the hepatic cells of rabbits has been observed (Kolmer, Brown, and Rule[28]). Nelson[29] has reported renal changes (degeneration of the tubular epithelium, tubular casts, and tubular dilatation), hemosiderosis of the spleen, degenerative changes in the testes and voluntary muscles, hypoplasia or hyperplasia of the bone marrow, and fatty changes in the liver. These changes occurred following the administration of fatal doses of sulfanilamide to rabbits. He also noted that the histologic changes were even more apparent in hens, in which there occurred marked fatty changes in the liver and kidney, hemosiderosis of the spleen and liver, peripheral neuritis, and hypoplasia of the bone marrow. On the contrary, other observers have been unable to find any pathologic lesions in either dogs or rats following sulfanilamide administration (Marshall, Cutting, and Emerson[30]). The discordance of opinion among the various workers mentioned may be due, in part, to differences of dosage, of methods of administration, of duration of administration, and finally, a variation in the type of animals used.

The mechanism of crystal deposition deserves some consideration at this point. Five possibilities present themselves: (a) Transfer of crystals from the inoculation site by phagocytes via the blood stream. (b) Accidental introduction of crystals into a subcutaneous blood vessel at the time of injection. (c) Spontaneous discharge of crystals into a blood vessel in the area of injection. (d) Accidental penetration of the peritoneal cavity at the time of injection, with deposition of crystals upon the surfaces of the liver and spleen. (e) Excessive concentration of sulfanilamide either in free or conjugated form in the blood, with subsequent crystallization into various tissues. The possibilities (a) and (d) can probably be excluded. We are unable to state, from the data available, the true explanation. Nor are we able to conclude that the crystals are those of free or conjugated sulfanilamide. Undoubtedly, however, they are related to the administration of this substance.

A further analysis of the tissue changes presented by these animals leads us to the conclusion that the activity of sulfanilamide, in this respect, can be divided into two main types: (1) a specific action, and (2) a nonspecific action. The specific action of sulfanilamide upon the recipient tissues is dependent upon its chemical structure. To this type of action can be attributed such tissue lesions as parenchymatous degeneration of hepatic and

renal epithelium, destruction of the red blood cells, and bone marrow alterations (hyperplasia, hypoplasia, and arrest of maturation of the myeloid series of cells). The nonspecific type of action is so called because we believe that it is analogous to that of any particulate substance, e.g., India ink or carbon particles. To this second type of action may be attributed the foreign body fibroblastic change at the injection site, the fibroblastic reactions at the sites of crystal deposition, the focal necrosis in the spleen and liver, and the hyperplasia of the reticulo-endothelial apparatus.

It would appear, from this study, that the factor of individual idiosyncrasy toward sulfanilamide might tend to enhance the specific rather than the nonspecific tissue effect. On the other hand, a diminution of the excretion of the substance might enhance either type of effect. Moreover, it is suggested that only the specific tissue effect would result from the oral administration of sulfanilamide, whereas the parenteral administration would be more prone to produce lesions in which both specific and nonspecific forms of activity of sulfanilamide are involved.

CONCLUSION

A study has been made of the effects of the prolonged administration of sulfanilamide upon the tissues of rats. It is suggested that the tissue changes resulting from sulfanilamide administration are of two types, one being dependent upon the specific chemical structure of the substance, and the other being characterized by a nonspecific foreign body response.

We wish to express our deep appreciation to Dr. J. L. Scianni, medical illustrator, University of Tennessee, College of Medicine, for the illustrations in this paper.

REFERENCES

1. Domagk, G.: Ein Beitrag zur Chemotherapie der bakterieller Infectionen, Deutsche med. Wchnschr. **61**: 250, 1935.
2. Tréfouël, J., Tréfouël, J., Nitti, F., and Bovet, D.: Activité du p-aminophénylsulfamide sur les infections streptococciques expérimentales de la souris et du lapin, Compt. rend. Soc. de biol. **120**: 756, 1935.
3. Goissedet, P., Despois, R., Gailliot, P., and Mayer, R.: De l'action du radical sulfamide: SO_2 NH_2 sur l'infection streptococcique expérimentale de la souris, Compt. rend. Soc. de biol. **121**: 1082, 1936.
4. Buttle, G. A. H., Gray, W. H., and Stephenson, D.: Protection of Mice Against Streptococcal and Other Infections by p-Aminobenzenesulphonamide and Related Substances, Lancet **1**: 1286, 1936.
5. Gley, P., and Girard, A.: Un nouveau dérivé de la sulfamido-chrysoïdine très actif contre l'infection streptococcique, Presse méd. **44**: 1775, 1936.
6. Colebrook, L., Buttle, G. A. H., and O'Meara, R. A. Q.: The Mode of Action of p-Aminobenzenesulfonamide and Prontosil in Haemolytic Streptococcal Infections, Lancet **2**: 1323, 1936.
7. Bliss, E. A., and Long, P. A.: The Activation of "Prontosil Solution" in Vitro by Reduction With Cysteine Hydrochloride, Bull. Johns Hopkins Hosp. **60**: 149, 1937.
8. Fuller, A. T.: Is p-Aminobenzenesulfonamide the Active Agent in Prontosil Therapy? Lancet **1**: 194, 1937.
9. Long, P. H., and Bliss, E. A.: Para-Aminobenzenesulfonamide and Its Derivatives, J. A. M. A. **108**: 32, 1937.
10. Raiziss, G. W., Severac, M., and Moetsch, J. C.: Chemotherapeutic Studies of Sulfamidyl in Experimental Beta-Hemolytic Streptococcic Infection, J. Chemotherapy **14**: 1, 1937.
11. Whitby, L. E. H.: An Experimental Assessment of the Therapeutic Efficacy of Amino Compounds, Lancet **1**: 1517, 1937.
12. Rosenthal, S. M.: Chemotherapy of Experimental Pneumococcus Infections, Pub. Health Rep. **52**: 48, 1937.

13. Tréfouël, J., Tréfouël, J., Nitti, F., and Bovet, D.: Chimiothérapie des infections streptococciques par les dérivés du p-aminophénylsulfamide, Ann. Inst. Pasteur **58**: 30, 1937.
14. Nitti, F., and Bovet, D.: Action du 4-sulfonamide-2-4-diaminobenzol (prontosil) sur des infections streptococciques de la souris provoquées par des streptocoques d'origine humaine, Compt. rend. Soc. de biol. **119**: 1277, 1935.
15. Long, Perrin H., Bliss, E. A., and Feinstone, W. H.: Mode of Action, Clinical Use and Toxic Manifestations of Sulfanilamide, J. A. M. A. **112**: 115, 1939.
16. Young, C. J.: Agranulocytosis and Para-Aminobenzenesulfonamide, Brit. M. J. **2**: 105, 1937.
17. Plumer, H. E.: Prontylin and Prontosil (Correspondence), New England J. Med. **216**: 711, 1937.
18. Berg, S., and Holtzman, M.: Fatal Granulocytopenia Following Sulfanilamide Therapy, J. A. M. A. **110**: 370, 1938.
19. O'Connell, J. T.: Sulfanilamide Poisoning: Fatal Case, U. S. Nav. M. Bull. **36**: 61, 1938.
20. Schwartz, W. F., Garvin, C. F., and Koletsky, S.: Fatal Granulocytopenia From Sulfanilamide, J. A. M. A. **110**: 368, 1938.
21. Shecket, H. A., and Price, A. E.: Fatal Granulocytopenia Following Administration of Sulfanilamide, J. A. M. A. **112**: 823, 1939.
22. Cline, E. A.: Acute Yellow Atrophy of the Liver Following Sulfanilamide Medication, J. A. M. A. **111**: 2384, 1938.
23. Ornsteen, A. M., and Furst, W.: Peripheral Neuritis Due to Sulfanilamide, J. A. M. A. **111**: 2103, 1938.
24. Buey, P. C.: Toxic Optic Neuritis Resulting From Sulfanilamide, J. A. M. A. **109**: 1007, 1937.
25. Geiling, E. M. K., and Cannon, Paul R.: Pathologic Effects of Elixir of Sulfanilamide (Diethylene Glycol) Poisoning, J. A. M. A. **111**: 919, 1938.
26. Hawking, F.: Pharmacological Actions of Sulphanilamide, Lancet **2**: 1019, 1937.
27. Hageman, P. O.: Toxicity of Sulfanilamide. A Study of the Pathological Lesions in White Mice, Proc. Soc. Exper. Biol. & Med. **37**: 119, 1937.
28. Kolmer, John A., Brown, Herman, and Rule, Anna M.: Toxicity, Therapeutic Activity, and Mode of Action of Sulfanilamide in Experimental Streptococcus Infections of Rabbits, J. Lab. & Clin. Med. **24**: 164, 1938.
29. Nelson, A. A.: Histopathological Changes in Hens and Rabbits Following Administration of Sulfanilamide and Sulfanilyl Sulfanilamide (Disulfanilamide), Pub. Health Rep. **54**: 106, 1939.
30. Marshall, E. K., Jr., Cutting, W. C., and Emerson, Kendall: The Toxicity of Sulfanilamide, J. A. M. A. **110**: 252, 1938

THE ACCURACY OF DIAGNOSIS OF APPENDICITIS*

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A STUDY was undertaken to determine primarily the accuracy of diagnosis of appendicitis at the Santa Monica Hospital. By "diagnosis" we refer, first, to the preoperative or clinical diagnosis; second, to the surgical or operative diagnosis; and third, to the postoperative or laboratory diagnosis. The first two diagnoses are made by the surgeon. The last is made by the pathologist and is our standard of accuracy.

By "appendicitis" we refer to inflammatory diseases of the appendix as they are commonly understood. We are aware that there is current considerable argument to the effect that removal of the pathologically normal appendix will afford relief from various symptoms in a high percentage of cases. Such claims, if true, may tend to minimize the importance of errors of a statistical nature. In this survey we are not directly interested in these considerations. We might suggest, in passing, that the occurrence of eosinophiles in some of these specimens might indicate the presence of an allergic reaction.

These appendectomies cover the ten-year period from January 1, 1928, to January 1, 1938. For purposes of discussion, classifications have been used as indicated by the headings in Table I.

In assembling the figures in this study an attempt has been made to verify the diagnoses as they appear on the charts. The operator may, through carelessness or for other reasons, introduce inconsistencies into his records. If his examination and findings clearly indicate this, the diagnosis is corrected accordingly. Surgeons often disregard the findings of the pathologist. Thus, one may make a clinical diagnosis of acute appendicitis and an operative diagnosis of acute or subacute appendicitis. The pathologist may diagnose normal appendix, while the surgeon signs the case out with a final diagnosis of acute or subacute appendicitis.

The reader will notice certain cases labeled "chronic appendicitis (I.A.)." Where the report of the pathologist is "chronic appendicitis," but his description (gross and microscopical) of the pathologic findings does not reveal definite pathology, the designation I.A. is added to indicate "innocent appendix." Many pathologic diagnoses of "chronic appendicitis" appear on charts as a carry-over from days of ultrascientific classification or as a euphemism. This policy has now been corrected in our laboratory. No appendix is noted as normal or innocent if its wall is thickened or stenotic, nor if any of them contain

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fecaliths. Appendices showing only some injection of the serosa or containing soft fecal material are classed as innocent.

No doubt a dozen different workers going over these same charts would get a dozen different sets of figures; but they would not vary much. Our results, working independently on separate groups, show that the pathologist (A. A. K.) considers three-quarters of the so-called chronic appendicitis specimens as innocent, whereas the other of us (A. S. A.) places only 2 out of 3 in that classification. Other percentages are practically identical.

We can see no advantage here in expressing percentages beyond the decimal and shall, therefore, use whole numbers only.

TABLE I

LABORATORY DIAGNOSIS

Showing the laboratory (pathologist's) diagnoses as compared to the clinical or preoperative diagnoses. (Thus, of the 142 cases diagnosed clinically as subacute, 17 were found to be subacute, 22 were chronic, etc. Similarly, of the 90 cases found pathologically to be subacute, 17 had preoperatively been diagnosed subacute, 18 had been diagnised as chronic, etc.)

CLINICAL DIAGNOSIS	ACUTE, NO RUPTURE	ACUTE, RUPTURE	SUBACUTE	CHRONIC	CHRONIC OBLITERATIVE	CHRONIC (I.A.)*	NORMAL	TOTAL (CLINICAL DIAGNOSIS)
Acute, not ruptured	604	37	48	62	27	101	97	975
Acute, ruptured	36	32	1	2	0	4	1	75
Subacute	14	1	17	22	7	59	22	142
Chronic	13	0	18	89	44	210	111	485
Normal (not mentioned)	5	1	6	30	36	80	263	421
Total (laboratory diagnosis)	672	71	90	205	114	453	493	2098

*Innocent appendix.

CLINICAL DIAGNOSIS

Acute (Including Ruptured) Appendicitis.—Of the 2,098 appendectomies, 1,050, or one-half, were diagnosed as acute. Of these, 709, or 67 per cent, were actually acute. This means that 2 appendices out of 3 will confirm a clinical diagnosis of acute appendicitis.

Conversely, we note that there were actually 743 cases of acute appendicitis in the entire series. Of these, 709, or 95 per cent, were diagnosed preoperatively as acute. This means that if one has acute appendicitis, the chances are 95 out of 100 that the physician will ultimately recognize it as such.

Significant (Acute and Subacute) Appendicitis.—There seems little use in considering the diagnosis of subacute appendicitis in the same way. We may, however, group acute and subacute appendicitis together, as including those cases in which there is definite and significant disease. Doing this, we find that of 1,192 clinical diagnoses of acute and subacute appendicitis, 790, or 66 per cent, were actually acute or subacute. Thus, if we diagnose significant pathologic change in the appendix, the chances are only 2 out of 3 that we will be right.

Contrariwise, we see that there were in the series 833 cases of acute and subacute appendicitis, of which 790, or 95 per cent, were diagnosed clinically as such. Here again, with significant pathologic change in the appendix, the chances are 95 out of 100 that the physician will correctly determine it. This indicates that our chief problem, statistically, lies not in recognizing acute or subacute appendicitis, but in recognizing those things with which they may be confused.

Chronic Appendicitis.—The field of chronic appendicitis involves much difference of opinion. Some claim that the entity does not exist. Some claim that it does exist, but should not include the obliterative types. So far as we are concerned, for these purposes, we shall group all cases of chronic appendicitis together, including with the normal cases those diagnosed as chronic but considered by us as having no pathology that could reasonably be supposed to give clinical symptoms, that is, those specimens designated by us as innocent appendices (I.A.).

The practical significance of obliterative appendicitis, however, may be indicated by Table II.

TABLE II

OBLITERATIVE APPENDICITIS

CLINICAL DIAGNOSIS		PATHOLOGICALLY OBLITERATIVE
Chronic appendicitis	485 cases	44 cases, or 9 per cent
Normal appendicitis	421 cases	36 cases, or 9 per cent

We see from Table II that there is the same percentage of obliterative appendices among "normal" individuals as among those thought to have chronic appendicitis. Therefore, we may suspect that the obliterative appendix causes no more trouble than the normal appendix.

We note in passing that obliterative appendicitis is not a well-recognized condition by the surgeon. Although it occurred 141 times, he mentioned it only 10 times.

Of the 485 diagnoses of chronic appendicitis, then, there were 133, or 27 per cent, that were actually sufficiently abnormal to justify the belief that they may have given rise to symptoms. Thus, the chances are about 1 out of 4 that a diagnosis of chronic appendicitis will be verified by the pathologist. It is true that occasionally a diagnosis of chronic appendicitis will lead to disclosure of an acute or subacute appendicitis. This elevates the level of accuracy of diagnosis to 34 per cent. This means that when we diagnose chronic appendicitis, the chances are only 1 in 3 that the appendix will be in any way abnormal.

Turning this around again, we note that of 319 cases of chronic appendicitis, 133, or 42 per cent, were diagnosed clinically as such. Thus the chances of our accurately recognizing chronic appendicitis clinically are 5 out of 12. Hence we are only slightly more likely to identify it when present than to diagnose it incorrectly when it is not present; and we are more likely to miss it than to recognize it when it is present.

In the "normal" group of 421 cases we find that the incidence of chronic appendicitis is about 16 per cent. We may thus conceive that of every 6 per-

sons on the street, 1 has chronic appendicitis from the standpoint of the pathologist. But the incidence of chronic appendicitis in those diagnosed as having it is only 27 per cent, or about 1 in 4. Our error would thus be fairly small if we would choose our cases of chronic appendicitis at random, without attempt at diagnosis.

We may put this another way. If the conditions found at operation upon the chronic appendicitis patient are the same as those found at operation upon many "normal" patients, then the so-called symptoms of chronic appendicitis may not arise from appendicitis, or even from the appendix, at all. Clinically, there may be no chronic appendicitis, or only a rare one. This fact will explain some of the conclusions to follow.

Interesting for comparison is Shelley's series[1] where, among 1,890 appendices removed from patients without sign or symptom of appendicial disease, two-thirds showed definite pathologic change.

Ruptured Appendicitis.—We feel that there may be little point in calculating the accuracy of diagnosis in cases of rupture. We suspect that neither surgeon nor pathologist goes to great pains to distinguish acute nonruptured from acute ruptured appendicitis, at times merely noting the acute form when rupture obviously existed. However this may be, our records show that of 75 clinical diagnoses of rupture, 61, or 81 per cent, were actually ruptured according to operative diagnosis and 32, or 43 per cent, according to laboratory diagnosis.

Conversely, of 118 cases diagnosed as ruptured operatively, 61, or 51 per cent, were so diagnosed clinically, while the laboratory confirmed the diagnosis in 64 cases, or 54 per cent of all cases.

Appendicitis of All Types (as a Group).—In this series of 2,098 appendectomies, 1,677 appendices, or 80 per cent, were diagnosed preoperatively as abnormal. This means that 1 appendectomy in 5 is an "incidental" appendectomy. Of the 1,677 diagnosed clinically as abnormal, 1,152, or 69 per cent, were recognized pathologically as abnormal. In other words, if we think that an appendix is abnormal in any way, the chances are about 2 in 3 that we are right. Of the 1,677 cases diagnosed as appendicitis clinically:

737, or 44 per cent were acute by laboratory examination.
84, or 5 per cent were subacute by laboratory examination.
253, or 15 per cent were chronic by laboratory examination.
603, or 36 per cent were normal by laboratory examination.

We may compare this to Aschoff's series[2] of 847 cases diagnosed as appendicitis with no evidence of it pathologically in 35 per cent. Sappington and Horneff[2] present an even worse picture with a series of 937 cases diagnosed as appendicitis and 565, or 60 per cent, pathologically normal. Other statistics present errors of 2 per cent, or even nothing at all.[3]

We may deduce from Table I that the chance of diagnosing "diseased appendix" incorrectly (36 per cent) is almost twice the chance of diagnosing "normal appendix" incorrectly (19 per cent). Thus it is twice as easy clinically for us to misdiagnose a normal appendix as a diseased one.

OPERATIVE DIAGNOSIS

Significant (Acute and Subacute) Appendicitis.—Referring to Table III, of 892 diagnoses of acute appendicitis made at the operating table, 711, or 80 per cent, were actually acute according to the pathologist. If we combine subacute with acute cases as previously, there were 1,090 operating room diagnoses of definite and significant appendicitis. The pathologist agreed in 797, or 73 per cent, of these cases. This means that for every 4 appendices that we diagnose at the operating table as being significantly diseased, we are wrong once. It is also interesting to note that of these 1,090 surgical diagnoses of acute and subacute appendicitis, 83 were chronic and 210 (1 in 5) were actually normal.

TABLE III

LABORATORY DIAGNOSIS

Showing the laboratory diagnoses as compared to the surgical or operative diagnoses. (Thus, of 198 cases diagnosed as subacute at the operating table, 28 were subacute, 30 were chronic, etc. Similarly, of the 91 cases diagnosed as subacute by the laboratory, 28 were thought to be subacute at the operating table, 20 were thought to be chronic, etc.)

OPERATIVE DIAGNOSIS	ACUTE, NO RUPTURE	ACUTE, RUPTURE	SUBACUTE	CHRONIC	CHRONIC OBLITERATIVE	CHRONIC I.A.	NORMAL	TOTAL (OPERATIVE DIAGNOSIS)
Acute, not ruptured	590	7	39	32	9	56	41	774
Acute, ruptured	50	64	1	2	0	0	1	118
Subacute	18	0	28	30	10	64	48	198
Chronic	11	0	20	125	57	285	199	697
Chronic obliterative	0	0	0	1	7	1	1	10
Normal	2	0	3	16	31	46	203	301
Total (Laboratory diagnosis)	671	71	91	206	114	452	493	2098

Conversely, we note that of the 742 cases of pathologically acute appendicitis, 711, or 96 per cent, were diagnosed as such operatively. Combining acute with subacute, of the 833 significantly inflamed organs, 797, or 96 per cent, were recognized. Thus our chances of correctly recognizing definite and significant inflammation of the appendix at the operating table are 96 out of 100.

Chronic Appendicitis.—Similarly, we diagnosed chronic appendicitis operatively 707 times when the condition existed 190 times, with an accuracy of 27 per cent, and we recognized chronic appendicitis 190 times out of 320 actual cases, with an accuracy of 59 per cent. Operatively, as clinically, we misdiagnose the normal appendix twice as readily as the diseased one.

Appendicitis of All Types (as a Group).—Of the 1,153 actually abnormal appendices, only 52 were diagnosed as normal at the operating table. But of the 945 normal appendices, 696 were diagnosed as abnormal. This means that our diagnosis of the pathologically normal appendix as made at the operating table is worthless and that we are more likely to be wrong than right by 3 out of 4. How much of this error is due to insincerity or carelessness and how much to inability must remain for each individual to decide. The error comes chiefly, of course, in the field of chronic appendicitis.

A reason for this lack of correlation between laboratory and operative findings becomes more clear when we study Table IV.

TABLE IV

OPERATIVE DIAGNOSIS

Showing the operative or surgical diagnoses as compared to the clinical diagnoses. (Thus, of the 140 cases diagnosed clinically as subacute, 83 were thought to be subacute at the operating table, 39 were thought to be chronic, etc. Similarly of the 198 cases thought to be subacute at the operating table, 83 had been diagnosed subacute clinically, 21 had been diagnosed chronic clinically, etc.)

CLINICAL DIAGNOSIS	ACUTE, NO RUPTURE	ACUTE, RUPTURE	SUB-ACUTE	CHRONIC	CHRONIC OBLITERATIVE	NORMAL	TOTAL (CLINICAL DIAGNOSIS)
Acute, not ruptured	736	51	82	56	1	49	975
Acute, ruptured	9	61	1	2	0	3	76
Subacute	13	2	83	39	0	3	140
Chronic	8	1	21	426	6	17	479
Normal (not mentioned)	5	3	11	184	4	221	428
Total (operative diagnosis)	771	118	198	707	11	293	2,098

Thus, with 1,191 cases diagnosed clinically as acute or subacute, 1,038 were so classified at the operating table, while only 790 were acute or subacute. With 479 diagnosed chronic clinically, 432 were diagnosed chronic operatively and 133 were actually chronic. With 428 presumed to be normal clinically (patient operated for other conditions entirely) 221 were diagnosed normal at operation, while 343 were actually normal.

Let us look at it this way:

Clinically *normal* 428	Operatively normal 221	Pathologically normal 343
Clinically *chronic* 479	Operatively normal 17	Pathologically normal 321

Whether the clinical diagnosis is normal or chronic, the chances of the appendix being actually normal are almost the same, while the chances of diagnosing the normal appendix incorrectly at operation are about 12 to 1 *in favor of the operator's preoperative diagnosis.* We may, therefore, conclude that the operator is governed mainly by his clinical diagnosis in distinguishing normal from chronically altered appendices, and that these two conditions are actually indistinguishable in the great majority of cases. The same holds true with other types of appendicitis, though to a lesser degree.

CAUSES OF ERROR

One cause of operative error has been discussed, namely, suasion of the operator in favor of his preoperative diagnosis. This factor is further evidenced, first, by the relative tendency to diagnose an appendix as normal rather than chronic when other pathology is present and, second, to make an intermediate diagnosis when the clinical diagnosis is wide of its mark.

Errors in clinical diagnoses of *acute appendicitis* occur mainly in females and are due to ruptured ovarian follicles and cysts, pelvic inflammatory disease,

ectopic pregnancy, and other gynecologic conditions. Of 61 cases operated upon for acute appendicitis, where a normal appendix was found at operation, 26 come in this group. Six were due to gall bladder disease, 7 to perityphlitis,* 2 to ruptured duodenal ulcer, 2 to intestinal obstruction, 2 to Meckel's diverticulitis, 1 each to lymphoma, gastro-enteritis, mesenteric adenitis, and coronary disease, and 12 to undetermined cause.†

The greatest cause of error in general appears to be nonsurgical conditions. Where *chronic* appendicitis is diagnosed alone clinically, and appendicitis is not discovered at operation, the great majority of cases will reveal no lesion to exploration as performed at this hospital. It may be noted, moreover, that the usual incision for chronic appendicitis, as done by the hospital staff, is not a muscle-splitting incision, but some form of rectus or midline incision.

A potent factor in misleading surgeons in diagnosis is undoubtedly their failure to possess accurate criteria of appendicitis. Reference is frequently made to veils, kinks, serosal injection, retrocecal position, etc., as evidencing appendicitis and justifying the diagnosis thereof.

MORTALITY

In collecting these statistics we have noted fatal cases, though this may not be directly in line with our survey. There were in all 26 deaths, distributed as follows:

Acute ruptured appendicitis	16
Acute nonruptured appendicitis	10
Appendix normal or innocent	9

Of the 9 persons who died from complications other than appendicitis, 2 were operated upon due to erroneous diagnosis of acute appendicitis. One was operated upon for chronic appendicitis and hemorrhoids, and an innocent appendix was found. The other 6 were "incidental" appendectomies, the patients dying of the primary lesion or a complication of it.

It will be noted that all but 1 of the deaths in operations for appendicitis, where no other serious condition existed, were due to *acute* appendicitis only. Mortality for operation itself has, therefore, been less than 1 in 2,000 . Mortality for acute appendicitis with rupture was about 1 in 7, using the operative diagnosis of rupture as a basis, whereas the mortality in acute nonruptured cases was about 1 in 67.‡ Our principal concern for improving the death rate is, therefore, with these 2 groups, and our principal concern in avoiding unnecessary operations has to do with morbidity and economy.

The total mortality in acute appendicitis has been reduced 65 per cent (from 8 per cent to 3 per cent) in the second five-year period as compared to the first five-year period (Table V), while appendectomies have increased twice as rapidly as the general population (50 per cent) and at the same rate as increase in staff membership§ (110 per cent) over the ten-year period. It may

*This term is used to indicate some type of inflammation in the region of the cecum and of unknown origin.

†Included here are some cases called appendicitis by the surgeon and one called spastic colitis.

‡Bower[3] states: "When the serous membrane is intact, 1 in 183 dies; when it is ruptured, 1 in 8 dies."

§Total staff membership 50 on January 1, 1928, and 105 on January 1, 1938.

well be that the doubling of operations for acute appendicitis accounts for the reduction in ruptures and mortality.

TABLE V

MORTALITY BY YEARS

YEAR	CASES OF PATHOLOGICALLY ACUTE APPENDICITIS (ALL TYPES)	TOTAL NUMBER OF DEATHS	NO. OF DEATHS WITH RUPTURE	PERCENTAGE OF DEATHS IN ACUTE CASES (ALL TYPES)
1928	43	1	0	2
1929	68	6	3	9
1930	69	5	2	7
1931	49	4	1	8
1932	67	7	6	10
1933	65	1	0	2
1934	60	0	0	0
1935	83	3	2	4
1936	120	5	1	4
1937	119	3	2	3

PROGRESS IN DIAGNOSIS

Mortality rates may be explained in part upon the basis of diagnosis. Thus, if diagnosis becomes more accurate, it may appear to decrease the mortality by adding cases of appendicitis formerly misdiagnosed, or it may increase the mortality by differentiating cases previously included through error. Actually, the clinical diagnosis of acute appendicitis for each of the ten years at this hospital has remained practically unchanged, as shown in Table VI.

TABLE VI

DIAGNOSIS OF ACUTE APPENDICITIS BY YEARS

Showing the accuracy of one type of diagnosis as compared with another for each of the ten years covered.

YEAR	DIAGNOSED CLINICALLY	CONFIRMED OPERATIVELY	% ACCURACY	DIAGNOSED CLINICALLY	CONFIRMED BY LABORATORY	% ACCURACY	DIAGNOSED BY LABORATORY	PREDICTED CLINICALLY	% ACCURACY	DIAGNOSED OPERATIVELY	CONFIRMED BY LABORATORY	% ACCURACY
1928	59	46	78	59	40	68	43	40	93	49	40	82
1929	105	84	80	105	66	63	68	66	97	87	65	75
1930	89	73	82	89	65	73	69	65	94	79	67	85
1931	68	58	85	70	48	69	49	48	98	59	49	83
1932	97	76	78	97	66	68	67	66	99	78	65	83
1933	93	73	79	93	64	69	65	64	99	76	64	84
1934	92	72	78	92	60	65	60	60	100	74	59	79
1935	113	96	86	113	78	69	83	78	94	100	80	80
1936	163	140	86	162	110	68	120	110	92	149	110	74
1937	170	139	82	170	112	66	119	112	94	141	112	80
Total	1,049	857	82	1,050	709	68	743	709	95	892	711	80

We do not know how many cases of appendicitis and nonappendicitis of various types were seen by the staff but neither operated upon nor even diagnosed. For this reason, we cannot determine all clinical error. Bedside research is badly needed for such information. We may infer a considerable delay somewhere from the fact that of 742 acute cases, 114, or 15 per cent (1 in 7), were ruptured at operation. However, 95 per cent of acute cases were ultimately

diagnosed as acute. The bulk of the remaining acute cases were diagnosed as appendicitis of some type, and operation was nearly always advised. Error in failure to *ultimately* recognize appendicitis must, therefore, be small. The tragedies come in delay. This delay may stem from our inability to recognize low-grade appendicitis accurately.

We note, however, the many diagnoses of acute appendicitis where acute appendicitis did not exist. The laboratory confirms our clinical diagnosis in only 2 cases out of 3. Until we can observe the appendix in the opened abdomen and correctly state whether or not it is significantly inflamed with an accuracy greater than 3 in 4, we may be unable to improve our recognition of the acutely inflamed appendix in the closed abdomen. Until our preoperative diagnosis does improve there must be unnecessary operations on the one hand, and on the other, unnecessary delay, ruptures, and deaths. Statistically, our diagnostic acuity has remained unchanged year after year (Table VI).

MULTIPLE DIAGNOSES

Special interest and importance attaches to those operations in which multiple clinical diagnoses are made. One wants to know whether the laws of chance justify a laparotomy, for chronic appendicitis especially, and whether fortuitous findings are likely to enhance the value of such surgery.

TABLE VII

MULTIPLE LESIONS

Showing the actual findings in operations for multiple lesions as compared to the clinical diagnoses. (Thus, of 226 cases diagnosed as having chronic appendicitis with other abdominal lesions, 8 had chronic appendicitis alone, 30 had no lesion at all, etc.)

CLINICAL DIAGNOSIS	ACTUAL FINDINGS (OPERATIVE AND LABORATORY)					
	CHRONIC APPENDICITIS WITHOUT OTHER LESIONS	CHRONIC APPENDICITIS WITH OTHER LESIONS	NORMAL APPENDIX WITHOUT OTHER LESIONS	NORMAL APPENDIX WITH OTHER LESIONS	ACUTE OR SUBACUTE APPENDICITIS WITH OR WITHOUT OTHER LESIONS	TOTAL (CLINICAL DIAGNOSIS)
Chronic appendicitis without other lesions	69	8	138	18	22	255
Chronic appendicitis with other lesions	8	56	30	128	4	226
Normal appendix (operation for other lesions)	5	62	21	322	13	423
Acute or subacute appendicitis (with or without other lesions)	97	22	206	77	792	1,194

In preparing a table of these cases (Table VII), only surgical conditions involving laparotomy are considered. The "actual findings" are taken from operative and laboratory diagnoses jointly, except for the appendix, where the laboratory diagnosis is accepted. Thus, conditions leading to no surgical specimen, but justifying surgery, are included in "other lesions" (than those in the appendix). Many of the conditions making up "other lesions" are rather trivial in some cases, but we believe it beyond the scope of this paper to evaluate

them. In some instances, as with small corpus luteum or other ovarian cysts, leading merely to puncture, the condition is disregarded.

We note that there were 255 cases with the clinical diagnosis of chronic appendicitis unaccompanied by any other diagnosis justifying laparotomy. Of this group:

(a) Diagnosis was exactly verified in 69 cases, or 27 per cent (about 1 in 4).

(b) Diagnosis of chronic appendicitis was verified but other lesions were found in 8 cases, or 3 per cent (about 1 in 32).

(c) Diagnosis was entirely in error but other lesions were found in 40 cases, or 16 per cent (about 1 in 6).

(d) There was no lesion whatever to justify the laparotomy in 138 cases, or 54 per cent (about 1 in 2).

Coming to the cases diagnosed as having chronic appendicitis accompanied by other intra-abdominal lesions (including hernias), we see that there were 226. Of these:

(a) Diagnosis was exactly verified in 56 cases, or 25 per cent (1 in 4).

(b) Chronic appendicitis alone was found in 8 cases, or 4 per cent (1 in 28).

(c) Diagnosis was in error but other lesions were found in 132 cases, or 59 per cent (about 6 in 10).

(d) There was no lesion whatever to justify the laparotomy in 30 cases, or 13 per cent (about 1 in 8).

It appears that we can recognize chronic appendicitis alone and chronic appendicitis accompanied by other intra-abdominal lesions with equal facility (1 in 4), and that the "other lesions" considered in each case are as likely to be missing when diagnosed as present as when not diagnosed (1 in 30).

When no chronic appendicitis exists, moreover, we are likely to find some other lesion in only 22 per cent, or 1 out of 5 cases, if we didn't expect one previous to operation. If we did expect another lesion previous to operation and failed to find the chronic appendicitis itself, our chances are 82 per cent, or 5 in 6 of finding another lesion.

Noteworthy is the fact that of those operations for chronic appendicitis alone, over half may be described as useless, whereas, if we think that other lesions accompany the chronically changed appendix, 1 operation in 8 may be designated as useless.

Noteworthy also is the fact that there are also almost as many diagnoses of chronic appendicitis with as without other abdominal lesion. This suggests that extra-appendicial lesions are not active in the production of appendicitis.

Turning to those operations where we believe or assume that the appendix is normal, we note that there are 423. It is actually normal in 343, or 81 per cent, of the cases (4 in 5). In 21 cases, or 5 per cent (1 in 20), no pathologic condition was found, i.e., the operation may be described as useless. As this error is less than that for the diagnosis of appendicitis as a whole, 36 per cent, it means that appendicitis is 7 times as easy to misdiagnose as other abdominal lesions (as a group).

One final observation: Of the 1,194 cases thought clinically to have acute or subacute appendicitis (with or without other lesions), but not having it, 99, or 8 per cent, had lesions elsewhere, while 97, or 8 per cent, had chronic appendicitis alone, and 206, or 17 per cent, had no intra-abdominal lesion whatever to justify laparotomy, so far as was found.

SUMMARY

Following are some of the conclusions resulting from this statistical study.

1. Only 2 appendices out of 3 will confirm a clinical diagnosis of acute appendicitis.

2. Of appendices actually acute, 95 per cent will ultimately be correctly diagnosed clinically.

3. The foregoing statements hold true when we group acute and subacute appendicitis together.

4. Chronic obliterative appendicitis causes no more trouble clinically than the normal appendix.

5. The chances are 1 out of 4 that a diagnosis of chronic appendicitis will be verified by the pathologist.

6. A diagnosis of chronic appendicitis means that the chances are 1 in 3 that the appendix is in any way abnormal.

7. When chronic appendicitis actually exists, the chances are about 5 in 12 that we will recognize it clinically; i.e., the chances are that we will not recognize it when we meet it.

8. One "normal" person in 6 has a pathologically "chronic appendicitis."

9. Thirty-five per cent (one-third) of all individuals operated upon for diseased appendix have a normal appendix.

10. It is twice as easy for us to misdiagnose a normal appendix clinically as a diseased one.

11. For every 4 appendices that we diagnose as significantly diseased (acute or subacute appendicitis) at the operating table, we are wrong once, and more than 1 such appendix in 5 will actually be normal.

12. Of appendices actually acutely or subacutely inflamed, 96 per cent will be recognized as such at the operating table.

13. When we think that an appendix is chronically inflamed at operation, we are correct in about 1 case in 4.

14. When we see a chronically inflamed appendix at the operating table, we can recognize it about 6 times out of 10.

15. Operatively, as clinically, it is twice as easy for us to misdiagnose the normal appendix as it is the diseased appendix, and when the appendix is normal our operative diagnosis will be wrong 3 times out of 4.

16. Whether the clinical diagnosis is "normal" or "chronic," the chances of the appendix being normal are almost the same, while the chances of diagnosing the normal appendix incorrectly at operation are about 12 to 1 in favor of the operator's preoperative diagnosis.

17. The chief cause of error in the clinical diagnosis of acute appendicitis is some gynecologic condition. Next in importance come "no surgical lesion," gall bladder disease, and inflammation in the region of the cecum, in that order.

18. Where chronic appendicitis is diagnosed alone clinically and not found, the great majority of cases will reveal no lesion to exploration.

19. The mortality in acute ruptured appendicitis over a ten-year period was 1 in 7 and that of the nonruptured acute cases was 1 in 67.

20. In ten years the diagnostic acuity of the staff of the Santa Monica Hospital has not changed, as regards appendicitis. Mortality rate has improved, along with increased frequency of operation.

21. Error in failure to ultimately recognize acute appendicitis is small (5 per cent). Delay in diagnosis and operation are the important factors. Because of it, 1 acutely inflamed appendix in 7 was ruptured at operation.

22. These statistics must be supplemented by data obtained before the patient's entry into the hospital to determine causes of delay, clinical error, etc.

23. We recognize chronic appendicitis alone and chronic appendicitis accompanied by other intra-abdominal lesions with equal facility, and accompanying lesions are as easily ruled out as recognized.

24. When we diagnose chronic appendicitis and it is absent at the operating table, we are likely to find some other lesion in only 1 case in 4 if we did not expect it before operation, and in 5 cases out of 6 if we did expect it.

25. Of those operations for chronic appendicitis alone, over half reveal no pathologic condition whatever. In operations for chronic appendicitis with other intra-abdominal lesions, 1 case in 10 reveals no pathologic condition.

26. Extra-appendicial lesions are not active in producing appendicitis.

27. When the appendix appears to be normal clinically (operation for other cause entirely), it is actually normal in 4 cases out of 5. In these cases, no pathologic condition at all was found in only 3 per cent.

28. Appendicitis is 7 times as easy to misdiagnose as other abdominal conditions (as a group).

29. Of cases thought clinically to have acute or subacute appendicitis, 1 case in 6 revealed no pathologic condition of any kind at operation.

REFERENCES

1. Shelley, H. J.: Chronic Appendicitis; Is It a Clinical Entity? Arch. Surg. **37**: 17, 1938.
2. Sappington, S. W., and Horneff, J. A.: Appendicitis From Histological Standpoint, Am. J. Surg. **39**: 23, 1938.
3. Bower, John O.: A Clinical Pathologic Classification of Acute Appendicitis and Peritonitis Complicating Perforative Appendicitis, Am. J. Surg. **45**: 67, 1939.

THE STABILITY OF ASCORBIC ACID IN BLOOD*

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WE HAVE been engaged in an extensive study of vitamin C metabolism in health and in disease, with particular reference to rheumatic fever and rheumatoid arthritis.[1, 2] In previous work we have shown that the plasma level of reduced ascorbic acid during fasting afforded an accurate index of the immediate nutritive status relative to vitamin C.[3] In this work we have employed the method of Farmer and Abt,[4, 5] and have investigated various factors which might influence the stability of vitamin C in the blood. This seemed particularly pertinent in view of the criticism of the method made by Pijoan and coworkers[6, 7] who reported that the ascorbic acid of the plasma undergoes rapid destruction after the blood has been withdrawn from the body. This was not in accord with our own experience. Pijoan also claimed that this loss could be prevented by the addition of potassium cyanide to the blood. Following this report we undertook a systematic study of the stability of ascorbic acid in blood and of the influence of cyanide upon this. While this work was in progress Farmer and Abt,[8] Friedman, Rubin, and Kees,[9] and Cushman and Butler[10] published reports which failed to show a stabilizing influence with cyanide. Furthermore, Farmer and Abt showed that potassium cyanide per se was capable of decolorizing 2,6 dichlorophenolindophenol and that it produced an increase in the apparent ascorbic acid values when added to blood. In the present paper further evidence bearing on the effect of potassium cyanide on 2,6 dichlorophenolindophenol and upon the stability of ascorbic acid in blood and plasma is reported. In addition, some experiments on the comparative stability of ascorbic acid in whole blood and plasma are presented.

Typical experiments showing the comparative stability of ascorbic acid in whole blood and plasma are shown in Table I. In these experiments 40 to 50 c.c. of blood were drawn from human subjects by venepuncture and distributed in 4 test tubes containing dry powdered sodium oxalate. Two of the portions of blood were centrifuged immediately, the plasma separated, and an aliquot portion of the latter was subjected to analysis as soon as possible. One test tube each of whole blood and plasma were then set aside at room and refrigerator temperatures, respectively. At the end of the time intervals indicated, analyses were carried out on the plasma of each of the 4 specimens. Deproteinization was carried out on 1 c.c. portions of each plasma according to the method of Pijoan and Eddy[11] and 2 c.c. portions of the filtrate were used for titration. The results recorded in Table I show that different bloods exhibit differences in their

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loss of reducing capacity on standing. In some cases the presence of red blood cells appears to exert a protective action on the ascorbic acid of the plasma, while in others this effect is absent. These results are somewhat at variance with those reported by Kellie and Zilva,[12] and Borsook and co-workers,[13] who reported that red blood cells exerted a protective influence on ascorbic acid. From one observation on dog blood, Barron, Barron, and Klemperer[14] concluded that erythrocytes do not exert any influence on the rate of oxidation of the ascorbic acid of the plasma.

TABLE I

THE STABILITY OF ASCORBIC ACID IN PLASMA AND WHOLE BLOOD AT ROOM AND REFRIGERATOR TEMPERATURE

TEMPERATURE		REFRIGERATOR		ROOM	
BLOOD SPECIMEN	TIME OF STANDING	PLASMA	WHOLE BLOOD	PLASMA	WHOLE BLOOD
B.	Hours	Mg. per cent	Mg. per cent	Mg. per cent	Mg. per cent
	Immediate	1.60	–	–	–
	0.6	1.41	1.60	1.38	1.60
	1.6	1.41	1.60	1.35	1.57
	3.0	1.41	1.60	1.23	1.54
	20.0	0.99	1.38	0.62	1.35
G.	Immediate	0.96	–	–	–
	1	0.96	0.96	–	0.96
	2	0.96	0.96	–	0.93
	3	0.93	0.96	–	0.90
	24	0.72	0.78	–	0.54
L.	Immediate	1.39	–	–	–
	0.5	1.30	–	1.30	–
	1.5	1.30	1.33	1.28	1.30
	3.0	1.30	1.30	1.28	1.28
	6.0	1.25	1.30	1.19	1.25
R.	Immediate	1.40	–		
	1	1.37	1.37		
	2	1.28	1.22		
	3	1.16	1.22		
	5	1.16	1.22		
	24	0.91	1.22		

However, the main point brought out by the data in Table I is the fact that whole blood kept at refrigerator temperature suffers but little loss in its ascorbic acid content over a period of two to three hours. We have examined some bloods which showed little or no loss under these conditions for periods as long as twenty-four hours. Cushman and Butler[10] have made similar observations.

After the appearance of Pijoan and Klemperer's paper we attempted to confirm their work. Since we had no potassium cyanide at hand, we employed sodium cyanide. Our observations showed no increase in the stability of the ascorbic acid of the plasma in the presence of sodium cyanide. Unlike potassium cyanide, sodium cyanide itself produced no decolorization of the dye 2,6 dichlorophenolindophenol. Three experiments were performed in which 40 to 50 c.c. of blood were drawn from three different individuals and 5 c.c. portions were distributed in tubes containing (1) sodium oxalate and (2) 15 to 20 mg. of a mixture of sodium oxalate and sodium cyanide in the proportion of two parts of the former and one of the latter. One sample of each series was centrifuged, and analyses were carried out on 1.0 c.c. quantities of the plasma according to the method described earlier. The remaining tubes of blood were kept at refrigerator

temperature (9° to 10° C.) and subsequently analyzed after varying intervals of time. In each case the time is reckoned from the moment the deproteinizing agent (metaphosphoric acid) was introduced into the plasma. The results of the experiments are shown in Table II. These are in accord with the observations of Farmer and Abt, Friedman, Rubin, and Kees, and Cushman and Butler. They show that the addition of sodium cyanide to blood offers no protection against the oxidation of the vitamin.

TABLE II

INFLUENCE OF NaCN ON THE ASCORBIC ACID VALUES OF PLASMA

(Whole blood left at refrigerator temperature)

BLOOD SPECIMEN	TIME OF STANDING	WITHOUT NaCN	WITH NaCN
R	Hours	Mg. per cent	Mg. per cent
	0	1.02	0.96
	2.5	0.96	0.96
	5.0	0.90	0.85
	24.0	0.79	0.79
G.	0	1.27	1.30
	2.5	1.21	1.24
	5.0	1.15	1.09
P	0	0.67	0.67
	2.7	0.60	0.60
	4.0	0.60	0.60
	27.5	0.43	0.49

Comparable studies were made with potassium cyanide. Ten milligram quantities of several lots of C.P. potassium cyanide dissolved in 2 c.c. of 2.5 per cent metaphosphoric acid were found to decolorize about 1.5 to 1.6 c.c. of dye with an ascorbic acid equivalency of 0.012 mg. per c.c. In addition, two other lots of sodium cyanide were tested in quantities 5 to 6 times as large as those reported for potassium cyanide, and the titration values were found to be of the same order of magnitude as blank determinations on equivalent volumes of metaphosphoric acid alone. When these larger amounts of sodium cyanide were used, much larger quantities of metaphosphoric acid were necessary because of the alkalinity produced by the former.

These tests led us to suspect that the samples of potassium cyanide might be contaminated with ferrocyanides. Ferrocyanide is listed as a common contaminant of both C. P. sodium and potassium cyanides. Moreover, ferrocyanides may be used in the preparation of cyanides. Tests on small amounts of potassium ferrocyanide dissolved in metaphosphoric acid showed this substance decolorized the "indicator reagent"; 0.86 mg. quantities of potassium ferrocyanide dissolved in 2 c.c. of 2.5 per cent metaphosphoric acid were found to decolorize 0.58 c.c. of dye (1 c.c. of dye = 0.222 mg. of ascorbic acid). Murray[15] lists 0.005 per cent as the maximum limit for ferrocyanides as an impurity of C.P. cyanides of sodium and potassium. Another common contaminant of cyanides is thiocyanate. This substance was found not to decolorize the dye used for the titration of vitamin C.

Further evidence that the decolorization of 2,6 dichlorophenolindophenol by potassium cyanide is attributable to the presence of ferrocyanides as an impurity was obtained by carrying out the Prussian blue test on both the sodium

and potassium cyanides which we had available. Five-tenths to 1 Gm. quantities of the different samples of potassium cyanide were found to give strong positive Prussian blue reactions, while similar quantities of the various sodium cyanides failed to give this test.

During the routine analysis of a large number of blood specimens we had observed that when an appreciable amount of hemolysis was present the ascorbic acid values were usually low. In this connection it has been reported by Gabbe,[16] Kellie and Zilva,[12] van Eekelen,[17] Emmerie and van Eekelen,[18] Berend and Fischer,[19] and Klodt,[20] that in the presence of oxyhemoglobin a part of the ascorbic acid is lost or destroyed. Fischer[21] has observed that the rate of disappearance of ascorbic acid in the presence of hemoglobin is extremely rapid. According to the work of Gabbe,[16] van Eekelen,[17] and Borsook and co-workers,[13] if whole blood or red blood cell suspensions are deproteinized with acid precipitants, little or none of the ascorbic acid which is present is found in the resulting filtrate. The release of hemoglobin from the red blood cells during the process of deproteinization appears to account for this change.

TABLE III

INFLUENCE OF HEMOLYSIS AND NaCN ON ASCORBIC ACID VALUES OF PLASMA

BLOOD SPECIMEN	TIME OF STANDING	PLASMA WITHOUT HEMOLYSIS	PLASMA + 0.1 C.C. HEMOLYZED BLOOD PER C.C.	
			WITHOUT NaCN	WITH NaCN
	Minutes	Mg. per cent	Mg. per cent	Mg. per cent
1	0	0.96	–	–
	5	–	0.73	0.73
	20	–	0.68	0.62
	40	0.90	0.62	0.57
	90	0.90	0.62	0.57
	120	0.85	–	–
2	0	1.10	0.75	0.75
	8	1.07	0.75	0.75
	20	–	0.70	0.70
	40	–	0.70	0.70
	60	0.93	0.64	0.64
	90	–	0.64	0.64
	120	0.87	0.58	0.58
	180	0.87	–	–

Relative to this, some studies were carried out to determine the effect of hemolyzed blood upon the ascorbic acid content of plasma. In conjunction with this, a study was made to ascertain whether cyanide prevented the loss or destruction of ascorbic acid which is brought about by hemolysis. The results of several such experiments are listed in Tables III and IV.

In carrying out these experiments blood was drawn from different individuals and collected in oxalated tubes. The plasma was separated and distributed in 3 or 4 tubes. Portions of the original blood or red corpuseles therefrom were hemolyzed with distilled water (one volume of blood or red blood cells to four volumes of water) and added to the plasma in the quantities indicated in the tables. Before the addition of the laked red blood cells sufficient distilled water was added to the control tubes and to the tubes which were to receive the lesser amounts of hemoglobin solution to adjust all preparations to equivalent dilution.

Analyses were carried out immediately after introduction of the hemolyzed blood cells and also after varying time intervals. One cubic centimeter aliquots of the mixtures were employed and the method used was the same as indicated earlier. In the instances where cyanide was tested a sufficient amount of this compound was added to give a concentration of approximately M/50. From an inspection of the data in Tables III to V it can be observed that hemolysis produces an instantaneous disappearance of ascorbic acid which is not prevented by the presence of sodium cyanide. This is followed by a slow decline in the concentration of the vitamin which also occurs in the specimens without hemolysis. The initial loss in ascorbic acid is also observed to increase with increase in the concentration of hemoglobin (Table IV). It is desirable here to point out that hemolysis must be avoided if reliable results are to be obtained for the reduced ascorbic acid values of plasma.

TABLE IV

INFLUENCE OF INCREASE IN HEMOLYSIS ON ASCORBIC ACID LOSS IN PLASMA

BLOOD SPECIMEN	TIME OF STANDING	PLASMA	PLASMA + 0.1 C.C. HEMOLYZED RED BLOOD CELLS	PLASMA + 0.2 C.C. HEMOLYZED RED BLOOD CELLS	PLASMA + 0.3 C.C. HEMOLYZED RED BLOOD CELLS
	Minutes	Mg. per cent	Mg. per cent	Mg. per cent	Mg. per cent
B	Immediate	1.27	1.06	0.94	0.80
	15	1.12	0.94	0.88	0.77
	30	1.12	0.97	0.85	0.68
	60	1.06	0.94	0.82	0.68
	120	1.00	0.94	0.82	0.71
G	Immediate	1.10	0.92	0.86	0.74
	15	–	0.92	0.86	0.74
	30	0.98	0.86	0.74	0.68
	60	–	0.86	0.74	0.68
	120	0.92	0.83	0.74	0.65

Further evidence that the "alleged" stabilization of ascorbic acid in blood by potassium cyanide is a mere artifact was obtained by carrying out parallel analyses on blood which had been treated with both sodium and potassium cyanide. The addition of cyanide in quantities equal to or greater than those recommended by Pijoan and Klemperer has been observed to produce hemolysis; this observation has also been reported by Farmer and Abt.[8] The degree of hemolysis has also been found to vary with the concentration of cyanide. Since hemolysis tends to decrease the indophenol titer of the blood, one would expect cyanide (above a certain limiting concentration) to produce such an effect. Actually that is what happens when sodium cyanide is used. However, when potassium cyanide is used, the reducing substance (probably ferrocyanide) which is present in the former as a contaminant tends to compensate in part for the ascorbic acid which is lost as a result of hemolysis. Table V summarizes the data obtained in two experiments in which both sodium and potassium cyanides were used.

In these experiments 50 c.c. quantities of blood were drawn from each of two subjects and placed in centrifuge tubes containing dry powdered sodium oxalate. The blood was centrifuged and the plasma was separated. The procedure at this point was somewhat different in each of the two experiments. In

Experiment 1, 10 c.c. portions of the plasma were placed in each of two test tubes. One cubic centimeter of distilled water was added to one 10 c.c. aliquot of the plasma and 1.0 c.c. of hemolyzed red blood cells to the other. The hemolyzed red blood cells were prepared by washing a portion of the centrifuged corpuscles twice with physiologic saline and adding 4 volumes of distilled water to one volume of corpuscles. Two cubic centimeter aliquot portions of each of these preparations were immediately transferred to centrifuge tubes containing the quantities of each cyanide listed in Table V. Analyses were carried out on these by a method which was essentially the same as that of Farmer and Abt,[5] differing only in the use of 10 per cent metaphosphoric acid instead of 5 per

TABLE V

INCREASE IN APPARENT ASCORBIC ACID BY KCN AND ABSENCE OF INHIBITION OF CYANIDE ON ASCORBIC DESTRUCTION DUE TO HEMOGLOBIN

EXPERIMENT NUMBER	CYANIDE ADDED	NO HEMOLYSIS	HEMOLYSIS
		Ascorbic acid mg. %	Ascorbic acid mg. %
1	None	0.94	0.61
	2 mg. NaCN	0.89	0.61
	4 mg. NaCN	0.80	0.58
	2 mg. KCN	1.09	0.83
	4 mg. KCN	1.23	1.06
2	None	1.28	0.67
	2 mg. NaCN	1.20	0.70
	4 mg. NaCN	1.17	0.70
	2 mg. KCN	1.45	1.14
	4 mg. KCN	1.56	1.23

cent metaphosphoric acid as a protein precipitant. The procedure used in the second experiment is as follows: 2 c.c. portions of the plasma were transferred to 10 centrifuge tubes containing the same quantities of sodium and potassium cyanide as was used in the first experiment. Two-tenths cubic centimeter quantities of hemolyzed corpuscles, prepared as described above, were added to each of 5 of the tubes, followed by 3.8 c.c. of distilled water and 4 c.c. of 10 per cent metaphosphoric acid. In setting up the remaining tubes for analysis the quantities of reagents were the same as recommended by Farmer and Abt. The analyses were carried out as soon as possible after the withdrawal of the blood. It is advisable to emphasize the fact that in the first experiment the hemolyzed corpuscles were added before the addition of the cyanide and in the second after the addition of the cyanide. The results show that the same effect is obtained regardless of whether cyanide is added to the plasma before or after the addition of the hemolyzed blood cells.

Although various explanations have been offered for the mechanism of the action of hemoglobin on the vitamin, it would not be desirable to do more than point these out, since we have no evidence to offer in this connection. Van Eekelen[17] and Kellie and Zilva[12] attribute the loss of ascorbic acid to oxidation of the latter by the oxygen of oxyhemoglobin. Klodt[20] is in accord with this, and claims to have shown that the loss of ascorbic acid parallels the oxygen saturation of the hemoglobin. On the other hand, Fischer, and also Gabbe, are not in agreement with this view. They believe that ascorbic acid is "bound adsorptively" by hemoglobin and does not disappear as a result of oxidation.

CONCLUSIONS

Whole blood left at refrigerator temperatures does not lose an appreciable amount of its ascorbic acid in two to three hours, and can be expected to give reliable analytical values within this period after withdrawal. In contact with corpuscles the average loss is only 12.6 per cent in twenty-four hours. There is no evidence to indicate that cyanide added to blood protects the vitamin from oxidation. Potassium cyanide itself decolorizes 2,6 dichlorophenolindophenol probably by virtue of the presence of ferrocyanide as an impurity. In the collection of blood for ascorbic acid determinations hemolysis must be avoided, since the release of hemoglobin from red blood cells produces a loss or destruction of the vitamin C of the plasma. The loss of vitamin C resulting from hemolysis is not prevented by cyanide.

REFERENCES

1. Rinehart, J. F., Greenberg, L. D., Olney, M., and Choy, F.: Metabolism of Vitamin C in Rheumatic Fever, Arch. Int. Med. **61**: 552, 1938.
2. Rinehart, J. F., Greenberg, L. D., Baker, F., Mettier, S. R., Bruckman, F., and Choy, F.: Metabolism of Vitamin C in Rheumatoid Arthritis, Arch. Int. Med. **61**: 537, 1938.
3. Greenberg, L. D., Rinehart, J. F., and Phatak, N. M.: Studies on Reduced Ascorbic Acid Content of the Blood Plasma, Proc. Soc. Exper. Biol. & Med. **35**: 135, 1936.
4. Farmer, C. J., and Abt, A. F.: Ascorbic Acid Content of Blood, Proc. Soc. Exper. Biol. & Med. **32**: 1625, 1935.
5. Farmer, C. J., and Abt, A. F., Determination of Reduced Ascorbic Acid in Small Amounts of Blood, Proc. Soc. Exper. Biol. & Med. **34**: 146, 1936.
6. Pijoan, M., Townsend, S. R., and Wilson, A.: Determination of Reduced Ascorbic Acid in Blood, Proc. Soc. Exper. Biol. & Med. **35**: 224, 1936.
7. Pijoan, M., and Klemperer, F.: Determination of Blood Ascorbic Acid, J. Clin. Investigation **16**: 443, 1937.
8. Farmer, C. J., and Abt, A. F.: Invalidation of Plasma Ascorbic Acid Values by Use of Potassium Cyanide, Proc. Soc. Exper. Biol. & Med. **38**: 399, 1938.
9. Friedman, G. J., Rubin, S. H., and Kees, W.: Effect of Addition of KCN to Whole Blood on Indophenol-Reducing Power of Plasma, Proc. Soc. Exper. Biol. & Med. **38**: 358, 1938.
10. Cushman, M., and Butler, A. M.: Use of Cyanide in the Determination of Ascorbic Acid, Proc. Soc. Exper. Biol. & Med. **39**: 534, 1938.
11. Pijoan, M., and Eddy, E.: Ascorbic Acid Content of Red Cells and Plasma, J. LAB. & CLIN. MED. **22**: 1227, 1937.
12. Kellie, A. E., and Zilva, S. S.: Catalytic Oxidation of Ascorbic Acid, Biochem. J. **29**: 1028, 1935.
13. Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C.: The Oxidation of Ascorbic Acid and Its Reduction in Vitro and Vivo, J. Biol. Chem. **117**: 237, 1937.
14. Barron, E. S. G., Barron, A. G., and Klemperer, F.: Studies on Biological Oxidations. VII. The Oxidation of Ascorbic Acid in Biological Fluids, J. Biol. Chem. **116**: 563, 1937.
15. Murray, B. L.: Standards and Tests for Reagents and C.P. Chemicals, New York, 1937, D. van Nostrand Company Inc.
16. Gabbe, E.: Adsorption of Ascorbic Acid in Blood, Klin. Wchnschr. **16**: 483, 1937.
17. van Eekelen, M.: The Amount of Ascorbic Acid in Blood and Urine. The Daily Requirements for Ascorbic Acid, Biochem. J. **30**: 2291, 1936.
18. Emmerie, A., and van Eekelen, M.: Determination of Ascorbic Acid in Blood, Biochem. J. **31**: 2125, 1937.
19. Berend, N., and Fischer, M.: Adsorption of Vitamin C. Modification of Tillmann's Method for the Determination of Ascorbic Acid in Colorless Fluids, Biochem. Ztschr. **291**: 221, 1937.
20. Klodt, W.: Influence of Ascorbic Acid on the Oxygen Dissociation in the Blood and the Chemical Course of Vital Oxidation Processes, Klin. Wchnschr. **15**: 1636, 1936.
21. Fischer, Maria: Behavior of Ascorbic Acid Toward Hemoglobin. Mechanism in Which Ascorbic Acid Is Bound in the Red Corpuscles, Biochem. Ztschr. **292**: 16, 1937.

THE TREATMENT OF ACUTE ATTACKS OF BRONCHIAL ASTHMA BY INTRAVENOUS INJECTION OF AMINOPHYLLIN*

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THE first example of the use of purine bodies for relief of asthma was the clinical observation that hot coffee would sometimes relieve an acute attack. This was known for years, and was noted by Sollmann[1] who states that the mechanism was probably "partly psychic and partly bronchial." As he points out, Pal[2] in 1912 noted that in guinea pigs, caffeine would relax bronchial spasm induced by peptone or muscarine; also Higgins and Means[3] in 1915 demonstrated that caffeine would cause bronchial dilatation in the human being, and Meyer[4] demonstrated that it stimulates the respiratory center.

Recently other purine compounds have been found of striking benefit in the relief of asthma. As early as 1931 Herrmann and Aynesworth[5] successfully treated a refractory asthmatic by intravenous injection of 0.48 Gm. of aminophyllin diluted to 10 c.c. About this time also van Leeuwen[6] suggested the use of the xanthine group for asthma. In 1936 Hajos[7] noted their benefits and suggested that theophyllin facilitated the absorption of adrenalin. Efron and Tuft, in the discussion of a review by Tuft and Brodsky,[6] mentioned favorable results in bronchial asthma from intravenous administration of aminophyllin. Green, Paul, and Feller[8] in 1937 reported 16 such cases, and showed that aminophyllin caused definite increase of vital capacity, and lowered venous and intrathecal pressure without affecting arterial pressure or pulse rate. In the same month, Herrmann and Aynesworth[5] reported a thorough and extensive review of the subject, and presented 16 persons on whom adrenalin had been ineffectual. These received 41 injections, 31 of which "afforded prompt, complete, and persistent relief." Two cases were unsuccessful. Mild reactions to the drug were common. They noted that intravenous administration of aminophyllin apparently restored sensitivity to adrenalin. An additional case has recently been reported by Brown[9] who believe that adrenalin reactivity may be so restored. No fatalities have been reported.

DATA

During a ten-month period from July 1, 1938, to May 1, 1939, 41 different patients with acute bronchial asthma were treated on the wards of the Fourth Medical Division of Bellevue Hospital. Of these 22 were found to be refractory to adrenalin, and their acute attacks were treated by intravenous injections of aminophyllin. The adrenalin used was a solution containing one part of adrenalin (as adrenalin chloride) in a thousand parts of physiologic solution of sodium chloride. It was administered subcutaneously in the usual manner. The

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TABLE 1

RESULTS IN ALL 22 PATIENTS

CASE	AGE	SEX	DISEASE DURATION	NUMBER OF INJECTIONS	RESPONSE TO 0.48 GM. AMINOPHYLLIN INTRAVENOUSLY	DURATION OF BENEFIT	TOXIC EFFECTS
1	38	M	4 years	4	Immediate	12–15 hours	Vomited
2	47	M	1 year	5	5 minutes	5–12 hours	Nausea
3	54	M	5 years	1	20 minutes	2 hours	None
4	63	M	7 years	1	15 minutes	2½ hours	None
5	28	F	5 years	2	Immediate	3–4 hours	None
6	33	F	16 years	1	Immediate	12+ hours	None
7	42	F	9 years	1	10 minutes	12+ hours	None
8	23	F	4 years	2	5 minutes	19–20 hours	Burning in chest and head
9	47	M	15 years	1	10 minutes	Slight 1 hour	None
10	21	F	3 years	35	5-30 minutes	Transient 5 minutes to 12 hours	Occasional nausea and vomiting
11	34	M	1 year	3	Immediate	4–8 hours	None
12	26	M	6 years	1	2 minutes	5 hours	Dizziness, retching and "pounding head"
13	39	M	9 years	3	5–10 minutes	3–10 hours	None
14	53	F	1 year	1	Immediate	3 hours	Vomiting
15	26	F	3 years	3	Immediate	1–2 hours	None
16	68	F	20 years	1	Immediate	No recurrence	None
17	43	F	5 years	2	Immediate	12–16 hours	Nausea and vomiting
18	29	F	Several	1	Immediate	No recurrence	Head and chest pains
19	41	F	8 years	1	10 minutes	No recurrence	None
20	36	M	Several	1	Immediate	No recurrence	None
21	73	F	Several	1	5 minutes	No recurrence	None
22	43	M	19 years	7	Immediate	12–36 hours	None

dosage, except where otherwise noted in the tables, was 0.5 c.c. No allergic studies were made on these cases. They were considered acute emergencies and treated as such. In all cases the aminophyllin was given undiluted, 0.48 Gm. in 2 c.c. of aqueous solution. The entire amount was administered intravenously within two minutes, a 25 gauge hypodermic needle preferably being used. About a third of our cases showed one or more of the following undesirable symptoms: dizziness, nausea, retching, vomiting, "pounding of the head," "burning in chest and head," or head and chest pain. No alarming effects were noted. No striking or permanent effects were noted in either pulse or blood pressure.

RESULTS

Twenty-two different patients were given a total of 78 injections of 0.48 Gm. or 2 c.c. of aminophyllin intravenously. No regard was given to body weight, age, or sex. Weights varied between 85 and 160 pounds, and ages from 21 to 73 years. Ten patients were males; 12 were females. The duration of disease in these patients varied from one to twenty years.

In half of the cases relief was immediate. The effect in others was evident in twenty to thirty minutes. The duration of benefit was from one hour to absence of recurrence over a period of days. In no case was it necessary to

give more than one injection within a period of less than two hours. These observations are shown in Table I.

We did not note any restoration of effectiveness of adrenalin, but can only conjecture that this may have happened in view of the number of persons that required only one injection, were subsequently discharged, and apparently did not have another refractory attack. Slightly more than one-half of our patients fall into this class.

TABLE II

PATIENTS RECEIVING INTRAVENOUS AMINOPHYLLIN ONLY

CASE	AGE	SEX	RESPONSE	NO. OF ATTACKS TREATED	TOXIC SYMPTOMS	DISEASE DURATION
1	34	M	Immediate	3	None	1 year
2	26	M	2 minutes	1	Dizziness, retching, "pounding head"	6 years
3	39	M	5–10 minutes	3	None	9 years
4	53	F	Immediate	1	Vomited	1 year
5	26	F	Immediate	3	None	3 years
6	68	F	Immediate	1	None	20 years
7	43	F	Immediate	2	Nausea and vomiting	5 years
8	29	F	Immediate	1	Head and chest pains	Several years
9	41	F	10 minutes	1	None	8 years
10	36	M	Immediate	1	None	Several years
11	73	F	5 minutes	1	None	Several years
12	43	M	Immediate	7	None	19 years

Those patients receiving intravenous injections of aqueous solution of aminophyllin for acute asthma without history of previous medication for the immediate attack are shown in Table II. In 8 of the 12 cases relief was immediate and striking. Four obtained relief within ten minutes. One-third had minor toxic symptoms. All stated that they experienced greater relief than that given by adrenalin on previous acute episodes.

TABLE III

PATIENTS RECEIVING INTRAVENOUS AMINOPHYLLIN FOLLOWING ADRENALIN

CASE	AGE	SEX	ADRENALIN 1:1,000 SUBCUTANEOUSLY	INTERVAL FROM LAST ADRENALIN	RESPONSE TO 0.48 GM. AMINOPHYLLIN INTRAVENOUSLY	TOXIC SYMPTOMS	DISEASE DURATION
1	38	M	0.5 c.c.	24 hours	Immediate	Vomited	4 years
2	47	M	0.5 c.c. (intravenously)	45 minutes	5 minutes	Nausea	1 year 5 years
3	54	M	1.25 c.c.	25 minutes	20 minutes	None	
4	63	M	0.5 c.c.	105 minutes	15 minutes	None	7 years
5	28	F	0.5 c.c.	6½ hours	Immediate	None	5 years
6	33	F	0.5 c.c.	12 hours	Immediate	None	16 years
7	42	F	0.5 c.c.	8 hours	10 minutes	None	9 years
8	23	F	0.5 c.c. 8 times	40 minutes	5 minutes	Burning in chest and head	4 years

In Table III are presented 8 patients who had been given adrenalin injections by the ambulance surgeon or admitting physician for their present attack

without relief. The interval between the adrenalin and the aminophyllin injections varied from twenty-five minutes to twenty-four hours. All patients were benefited. Three of them obtained good results immediately; the others, in twenty to fifty minutes. Three had minor toxic symptoms.

TABLE IV

ATYPICAL CASES

CASE	AGE	SEX	RESPONSE TO 0.48 GM. AMINOPHYLLIN INTRAVENOUSLY	NO. OF ATTACKS TREATED	TOXIC SYMPTOMS	REMARKS
1	47	M	10 minutes, slight	1	None	Response not striking
2	21	F	Varies from 5 minutes to half hour	35	Occasional nausea and vomiting	Originally effective but relief now only transient

The two patients in Table IV are classed by themselves. The first was a 47-year-old white man whose history was of fifteen years' standing. His benefit was only slight after ten minutes, and lasted only one hour. His clinical picture was complicated by marked emphysema and syphilitic heart disease. His asthma was of an allergic type.

The second case was that of a 21-year-old white woman who had been under almost constant treatment for the previous three years. Having been found allergic to many substances, she was not benefited by desensitization, removal of recurrent nasal polyps, or various other nose and throat procedures. Even irradiation of her sinuses, hilar regions, and blocking of her stellate ganglia offered no relief. Being accustomed to giving herself adrenalin (as much as twenty 0.5 c.c. injections of 1:1,000 solution per day), she became refractory to it. Amyl nitrite inhalations and nitroglycerin sublingually afforded only very transient relief. Emaciation and emphysema were marked. When she first received intravenous aminophyllin, nine months ago, the relief was striking and lasted several days. In the course of her disease she received a total of 35 injections. While they still afforded her some benefit, it was more transient. A complicating factor was the fact that she had few available veins, and subsequent injections had to be intramuscular; they were painful and definitely less effective.

SUMMARY

1. Perusal of the literature reveals that during the past nine years acute asthmatic attacks have been successfully treated by intravenous injections of aminophyllin. At least 33 cases are reported in the American literature with no fatalities.

2. During a ten-month period this drug has been extensively used for such cases on the Fourth Medical Division of Bellevue Hospital with very favorable and immediate results.

3. Twenty-two persons with severe bronchial asthma received a total of 78 injections of aminophyllin. Twenty gave almost immediate favorable response. Two responded less favorably. Eight, or 36 per cent, were long-standing asthmatics who had been treated with the usual adrenalin injections,

but whose attacks persisted unabated. Twelve persons received aminophyllin alone for their present attack with almost immediate favorable response.

4. All cases were to varying degrees refractory to adrenalin.

5. Eight cases showed minor toxic symptoms. No untoward effects were noted.

CONCLUSIONS

1. Aminophyllin, when given intravenously, is a most efficacious drug in the treatment of intractable acute attacks of bronchial asthma.

2. It is especially useful in cases which have become refractory to adrenalin.

NOTE: Since acceptance of our paper, the subject has also been considered by the following authors, with no untoward results noted:

Efron, B. C., and Everett, P.: Theophylline Ethylenediamine (Aminophyllin) in Bronchial Asthma, New Orleans M. & S. J. **92**: 77, 1939.

Keeney, E. L.: The Medical and Surgical Treatment of Severe Bronchial Asthma, Bull. Johns Hopkins Hosp. **66**: 34, 1940.

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REFERENCES

1. Sollmann, T.: A Manual of Pharmacology, Philadelphia, 1932, W. B. Saunders Co., p. 275.
2. Pal, J.: Caffein, Deutsche med. Wchnschr. **38**: 1774, 1912. Quoted by Sollmann.
3. Higgins, H. L., and Means, J. H.: Respiration, J. Pharm. **7**: 1, 1915. Idem.
4. Meyer, F.: Caffein, Arch. f. Physiol. **1**: 1, 1915. Idem.
5. Herrmann, G., and Aynesworth, M. B.: Successful Treatment of Persistent Extreme Dyspnea "Status Asthmaticus," J. LAB. & CLIN. MED. **23**: 135, 1937.
6. Tuft, L., and Brodsky, M. L.: The Influence of Various Drugs Upon Allergic Reactions, J. Allergy **7**: 238, 1936.
7. Hajos, K. : Observations on Therapy in Asthma, Wien. klin. Wchnschr. **49**: 737, 1936. Quoted by Brown, reference 9.
8. Green, J. A., Paul, W. D., and Feller, A. E.: The Action of Theophylline With Ethylenediamine, J. A. M. A. **109**: 1713, 1937.
9. Brown, G. T.: Aminophyllin in Asthma, J. Allergy **10**: 64, 1938.

LABORATORY METHODS

A NOTE ON THE DETERMINATION OF TOTAL SERUM PROTEINS, SERUM ALBUMIN, AND SERUM GLOBULIN*

IDA KRAUS, PH.D., CHICAGO, ILL.

THE following is a modified procedure adapted from the methods of Campbell[1] and Koch.[2] Campbell's use of sodium sulfite at room temperature to precipitate serum globulins instead of sodium sulfate at 37° C. (the method of Howe[3]) is particularly valuable for the clinical laboratory where the facilities are limited. We† have tried the two methods in duplicate on 25 different human sera, and found that the differences between the two methods were well within the experimental error of the duplicate determinations of each method.

The procedure is now employed at the Cook County Hospital. The total nitrogen is determined in a diluted serum; the nonprotein nitrogen, in a trichloracetic acid filtrate; the albumins (plus N.P.N.), in the filtrate from the globulins precipitated by sodium sulfite. From these data the total serum proteins, serum albumins, and globulins are calculated.

The reagents used include:

1. Sodium chloride 0.9 per cent.
2. Sodium sulfite 21 per cent.
3. Trichloracetic acid 5 per cent.
4. Sulfuric acid 1:1.
5. Hydrogen peroxide, 30 per cent reagent grade.
6. Nessler-Folin reagent (Koch modification).
7. Standard ammonium sulfate solution (5 c.c. = 0.15 mg. nitrogen).

PROCEDURE

Total serum proteins (albumins plus globulins): Pipette accurately 0.5 c.c. of serum into a 25 c.c. graduate cylinder or volumetric flask and make up to volume with 0.9 per cent of sodium chloride. Mix well by inversion and transfer 1 c.c. of the solution to a 1 by 8 inch pyrex test tube for a micro-Kjeldahl determination.

Albumins: Pipette 0.5 c.c. of serum into a test tube and add from a burette or pipette 9.5 c.c. of 21 per cent sodium sulfite. Mix by inversion and allow to stand at room temperature for ten minutes or longer. Filter through a twice-folded 7 cm. Whatman No. 40 filter paper. At first the solution comes through turbid, but by refiltering a water clear filtrate is obtained (if necessary add a knife edge of pumice). Transfer 0.5 c.c. of the *clear filtrate* to a 1 by 8 inch pyrex test tube for a micro-Kjeldahl determination.

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†Dr. Homer Weir and myself.
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Nonprotein Nitrogen: Pipette accurately 1 c.c. of serum into a 1 by 8 inch test tube or a 50 c.c. Erlenmeyer flask and measure accurately from a burette 14 c.c. of 5 per cent trichloracetic acid. Mix well and filter. Use 4.5 c.c. of the clear filtrate for a micro-Kjeldahl determination.

Micro-Kjeldahl: To each tube add 1 c.c. of 1:1 sulfuric acid and a bead, and evaporate off the water by heating on a sand bath until dense white fumes of sulfuric acid fill the tube. Remove the tube and cool for thirty seconds. Then add 1 or 2 drops of 30 per cent hydrogen peroxide. Heat over the free flame for one minute. Usually the fluid remains water clear, but in case a discoloration appears, repeat the treatment with hydrogen peroxide and reheat for one minute. Treat blanks on all reagents similarly. Allow the tubes to cool and add 34 c.c. of distilled water. Stopper and mix by inversion, add 15 c.c. of Nessler-Folin reagent. Mix, let stand five minutes or more, and compare in colorimeter with the standard. The most convenient standard was found to be 5 c.c. ammonium sulfate* (equals 0.15 mg. N) plus 1 c.c. of 1:1 sulfuric acid, and 29 c.c. water. Mix and add 15 c.c. Nessler's reagent.

Calculations.—

a. *Total nitrogen:* $\frac{\text{Standard reading}}{\text{Unknown reading}} \times \text{Conc. standard (0.15 mg.)} \times \frac{25}{1.0} \times \frac{100}{0.5} =$ Mg. N in 100 c.c. of serum.

b. *Albumin plus N.P.N.:* $\frac{S}{U} \times \text{Conc. standard} \times \frac{10}{0.5} \times \frac{100}{0.5} =$ Mg. albumin plus N.P.N./100 c.c. serum.

c. N.P.N.: $\frac{S}{U} \times \text{Conc. standard} \times 4.5 \times \frac{100}{1} =$ N.P.N./100 c.c. serum.

(If 50 or less, the correction is insignificant and can be omitted.)

Changing N to % of protein:

$(a - c) \times \frac{6.25}{1000} =$ % total protein.

$(b - c) \times \frac{6.25}{1000} =$ % albumin.

% total protein − % albumin = % globulin.

REFERENCES

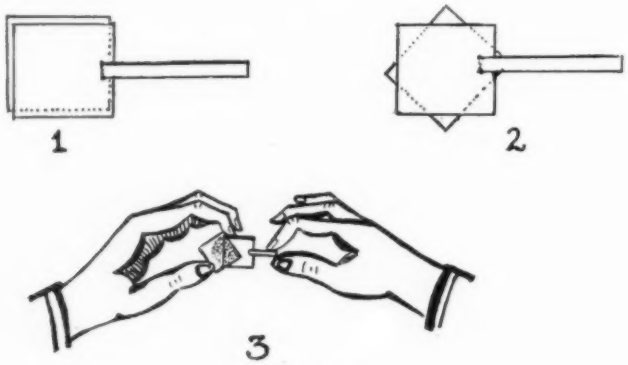
1. Campbell, W. R., and Hanna, M. I.: J. Biol. Chem. **119**: 15, 1937.
2. Koch, F. C.: Practical Methods of Biochemistry, ed. 2, Baltimore, 1937, William Wood & Co.
3. Howe, P. E.: J. Biol. Chem. **49**: 109, 1921.

*If too dark or too light, make up another standard using more or less ammonium sulfate and more or less water, the other additions remaining the same.

A SIMPLE AID IN MAKING A BLOOD SMEAR

BERNARD SILLS, MEXICO, D. F.

WHEN making blood smears with the "Ehrlich two-cover glass method" one encounters discouraging difficulties that compel one either to abandon the method and resort to one less reliable, or to devote a special amount of time to acquiring the technique. I have found that by using a simple device many of the difficulties can be overcome. The hands need not come in contact with the cleaned cover glasses until after the drop of blood has spread between their surfaces. The body of the familiar wax match used in Mexico serves satisfactorily for the wax stick.



Figs. 1-3.

One places two perfectly clean cover glasses face to face, so that their working surfaces are in contact, and then gently warms the tip of a wax stick until the wax just begins to melt. (This is done by passing the stick once or twice above the flame of a match.) The tip is then placed in contact with the edge of the top cover glass and held in place until the wax hardens (about 15 seconds), thus fixing the wax stick to the top cover glass (Fig. 1).

A drop of blood is obtained by pricking the finger or lobe of the ear. The top cover glass is lifted by means of the newly attached handle, and is touched to the drop of blood. It is then placed upon the other cover glass in such a manner that none of the corners coincide (Fig. 2). Contact causes the drop of blood to spread. Before the blood coagulates, the adherent cover glasses are lifted by the wax handle and are pulled apart by grasping the outer corners of the under glass with one hand and the wax handle with the other (Fig. 3). After drying, a gentle twist will detach the wax stick.

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AN EVALUATION OF METHODS FOR DETERMINING BLOOD AND URINARY AMYLASE*

DANIEL L. DOZZI, M.D., PHILADELPHIA, PA.

EVANS[1] (1912), Fearon[2] (1918), Elman and McCaughan[3] (1927), Myers and Reid[4] (1933), Pickford and Dorris[5] (1934), Somogyi[6] (1938), and others have made comparisons of the various methods employed for the quantitative estimation of amylase in blood and urine. Despite these investigations, there is no uniformity of opinion regarding which method or methods should be adopted for general use. The present study was undertaken in an attempt (a) to evaluate the methods from the standpoint of adequacy; (b) to observe the limitations of and the objections to each method; (c) to determine whether the same method is adaptable for the study of both blood and urine.

METHODS

Three basic methods are generally employed for the quantitative estimation of amylase in blood and urine. The method proposed by Wohlgemuth,[7] frequently called the iodine method, is based upon the disappearance of the iodine-starch blue color as the enzyme is producing hydrolysis of the starch. The second method, referred to as the saccharogenic method, has many modifications. It depends upon the determination of the amount of copper-reducing sugar formed by the enzymatic breakdown of starch. The third method, known as the viscosimetric method, relies upon the diminution in viscosity of a starch-enzyme mixture as scission of the starch particles is taking place.

The iodine method selected in the present study for both blood and urine was the Somogyi[6] modification of the Wohlgemuth test. Somogyi[6] has shown that the efficiency and accuracy of the method are increased by adopting time as the object of measurement. For the saccharogenic method, the procedure outlined by Somogyi,[8] which involves the Shaffer-Hartman copper-reduction method, was followed in estimating the amount of amylase in serum. The method I had adopted in another study[9] was used for determining the urinary amylase. The method involves the reduction of copper after the urine had been freed from interfering nitrogenous substances. The viscosimetric method, as employed by Elman and McCaughan,[3] was used for both urine and blood.

PROCEDURE

Healthy female dogs were selected, placed in metabolism cages, and maintained on the same diet throughout the experiment. Twenty-four-hour collections of urine were made by catheterizing at the beginning and at the end of the twenty-four-hour period. The urine obtained by the catheterization at

*From the Harrison Department of Surgical Research and the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia.
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the end of twenty-four hours was added to that collected from the metabolism cage. Blood samples were obtained by puncture of the jugular vein. In conjunction with other experiments, the results of which will be presented in another report, the opportunity was afforded to evaluate these methods during the normal fluctuations of amylase in the healthy dog and in dogs submitted to pancreatectomy, ligation of pancreatic ducts, administration of chloroform, and following renal injury produced by the administration of uranium nitrate.

In order that the different methods would be subjected to exactly the same conditions, the different tests were run simultaneously on both serum and urine in the same water bath at a temperature of 40° C. ± 0.5° C. The duration of incubation varied with the particular method, employing the same starch, electrolyte, and phosphate solutions.

A soluble potato starch was prepared according to the method of Small[10] and buffered to a pH of 6.8 with a phosphate solution; 5.84 Gm. of sodium chloride per 1,000 c.c. of prepared starch solution were then added. The strength of the starch solution differed with each method as follows: iodine method (0.4 per cent), saccharogenic method (2 per cent), and the viscosimetric method (7 per cent).

The amount of urine and serum used in each test also varied with the particular method in the following manner: iodine method, 1 c.c. serum, 4 c.c. urine; saccharogenic method, 0.5 c.c. serum, 5 c.c. urine; viscosimetric method, 0.4 c.c. serum, 2 c.c. urine. On the whole, each method studied was performed as advocated by the proponent of the particular method, with the exception of the starch solution and the quantity of enzyme solution used. Somogyi[6] used corn or rice starch in preference to potato starch. I have found potato starch entirely satisfactory. The quantities of enzyme solution were reduced because the present studies were conducted on dogs, and it is known that more amylase is found in the blood or urine of the dog than in man.

RESULTS

In general, all the methods studied were adequate for detecting a major increase or decrease in the amount of amylase in either blood or urine. However, in certain conditions, which will be pointed out later, one method proved to be more accurate than another. When studying the amylase content of serum, the methods tended in general to parallel one another qualitatively, but there is considerable quantitative difference. A typical example of the quantitative variation is shown in Fig. 1. The curves in Fig. 1 were obtained by calculating the amount of amylase per 100 c.c. of serum and expressing it in terms of units of amylase, or milligrams of glucose according to the particular method, then plotting the per cent rise or drop of amylase following certain procedures, using the first determination for the base line. The zero point on Fig. 1, therefore, does not indicate the absence of amylase. The per cent quantitative variation is the most marked for the Wohlgemuth method and the least marked for the viscosimetric method. The variation in the quantitative expression for each method may support the theory advanced by Crandall,[11] that the enzyme which produces changes in viscosity may be unlike the enzyme which promotes the

formation of copper-reducing sugar from the hydrolysis of starch. Furthermore, this may substantiate the view taken by Fearon,[2] that each method depends upon different phenomena. Despite the possibility that one may be studying different enzymes, it would appear that when studying urine an iodine method would always display greater fluctuations. On the other hand, since the duration of incubation and the quantity of urine and serum used with each test varied with the method, it is likely that some of the quantitative differences merely indicates that one method represents the optimum of enzyme activity while another is carried further to the point where the curve of enzyme activity begins to level off.

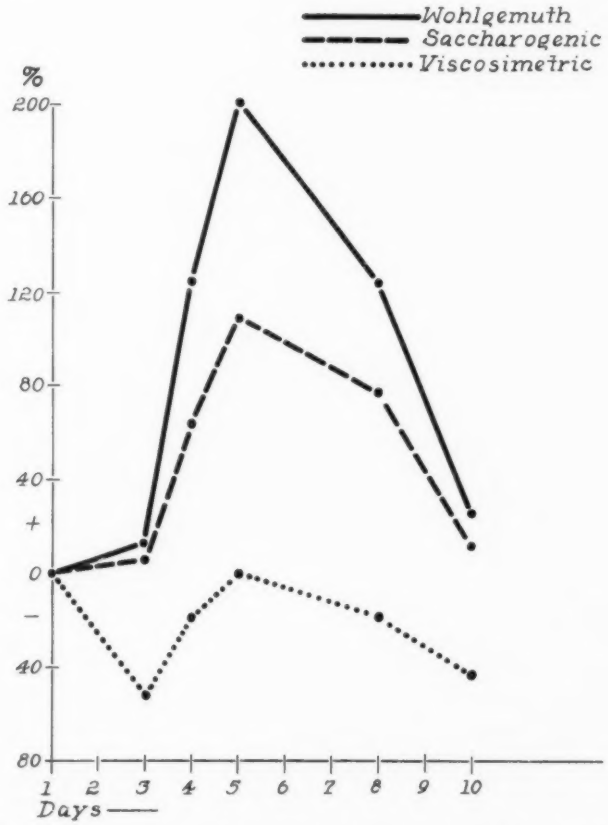

Fig. 1.—Comparison of the methods used when studying serum.

The saccharogenic method was entirely satisfactory, except when studying the dogs with diabetes, in which case it was necessary to resort to smaller quantities of serum. If there is marked hyperglycemia, one has either to use great dilutions or to resort to some procedure which will eliminate the excess sugar. In either event the method would not be desirable. In general, the saccharogenic method is more accurate in that it eliminates the difficulty of detecting small color changes and is entirely applicable even if the serum is discolored by bilirubin or some other pigment. Also it excludes the possibility of decolorization of the iodine in cases with azotemia.

The iodine method was quite satisfactory, except in the icteric dogs, at which time it was impossible to interpret the color changes. Occasionally with any sample, it was difficult to obtain a clear-cut end point. The viscosimetric method was satisfactory from the standpoint of simplicity and ease of performance. However, a serious objection was noted in that ofttimes an immediate, appreciable drop in viscosity was recorded when the starch solution and serum were brought together, which was independent of enzyme action, for the same result was obtained after the serum had been heated sufficiently to destroy the enzyme. Somogyi[6] has recorded this same source of error and has taken pains to demonstrate that the phenomenon is independent of enzyme action.

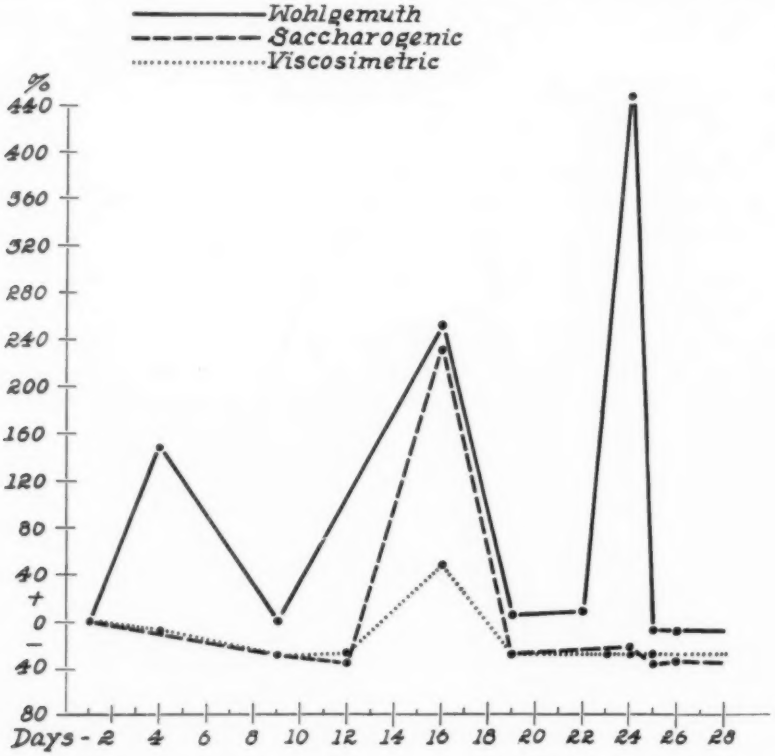

Fig. 2.—Comparison of the methods used when studying urine.

Elman and McCaughan[3] have also noted this error, but felt that the error was not great enough to make the method objectionable. Elman and McCaughan[3] have objected to the iodine and saccharogenic methods on the grounds that "a long chain of separate reactions occurs in the diastatic formation of sugar from starch, and the relative activity of the enzyme toward the different parts of the chain remains unknown" when selecting an arbitrary period of time for measurement. This objection is overcome by the modification of the iodine method used for this study wherein the progress of the amylase activity is observed at frequent intervals. One could easily do the same with the saccharogenic method by setting up a series of tubes and allowing each to incubate for different periods of time thereby determining the amylase activity for any

desired interval rather than adopting an arbitrary period of time. However, this does not seem necessary.

What has been said in the foregoing discussion concerning the methods when used for studying serum, applies, to some extent, when used with urine. The methods usually parallel one another qualitatively, as they did with serum. However, occasionally the iodine method did not follow the other two methods. A typical example of this deviation is shown for twenty-four-hour urines in Fig. 2, where it will be noted that the iodine method may indicate the presence of a large amount of amylase, whereas the other two methods show a low amylase content. This, undoubtedly, is due to decolorization of the iodine by the urine. Many workers have pointed out that all urines are capable of decolorizing iodine to a certain extent and consequently the iodine method is very unreliable with urine. The viscosimetric method is also unsuitable with urine because the initial reading following the addition of urine is practically always materially altered, necessitating frequent corrections of the "zero point." The saccharogenic method, although time-consuming, is entirely satisfactory with the same exception found when studying serum, in that it is difficult to employ if there are large quantities of sugar in the urine. While greater errors were noted when studying urine, the urine studies yielded no information that could not be obtained from the serum studies.

SUMMARY AND CONCLUSIONS

Iodine, saccharogenic, and viscosimetric methods were compared in both blood and urine of dogs that had had various procedures performed for producing rises and falls in amylase.

With all these methods one could detect in general appreciable changes in amylase content of blood or urine. However, there were considerable variations in the results. I conclude that while no one method is entirely satisfactory under all circumstances, the saccharogenic method is the most accurate and has fewer objectionable features than any other method studied. This method, though generally satisfactory, is difficult to employ when there is marked hyperglycemia or glycosuria. The method is equally applicable for studying blood or urine. The Wohlgemuth method is satisfactory for studying serum but is unreliable for studying urine. The viscosimetric method, being attractive for its simplicity and ease of performance, is suitable for studying serum except for an objection which has been pointed out. This method is not satisfactory for studying urine because of the necessity of frequent corrections of the "zero point."

Our observations confirm the opinions of Evans,[1] Fearon,[2] Myers and Reid,[4] and Somogyi[6] in that the copper-reduction method for estimating amylase is the most accurate.

Since all methods are less satisfactory with urine and urine yields no additional information, we question the advisability of performing routine urine studies. In order to have accurate data, the tests on urine must be performed on twenty-four-hour collections as was pointed out in another study,[9] a factor which introduces many difficulties. I studied[9] the normal fluctuations in urinary amylase in the healthy human being and found that the fluctuation

from individual to individual and in the same individual from day to day was so great that the interpretation of results is difficult. It is my belief that discrepancies in reports from different laboratories are due to normally wide ranges rather than to an inadequacy in methods for estimating amylase.

REFERENCES

1. Evans, C. L.: A Criticism of Two Methods for the Determination of Amyloclastic Activity, J. Physiol. **44**: 220, 1912.
2. Fearon, W. R.: The Amylolytic Properties of Urine and the Significance of Variations in Health and Disease, Dublin J. Med. Sc. **146**: 149, 1918.
3. Elman, R., and McCaughan, J. M.: The Quantitative Determination of Blood Amylase With the Viscosimeter, Arch. Int. Med. **40**: 58, 1927.
4. Myers, V. C., and Reid, E.: Comparison of Several Different Methods for Quantitative Estimation of Amylase, J. Biol. Chem. **99**: 595, 1933.
5. Pickford, G. E., and Dorris, F.: Micro-Methods for the Detection of Proteases and Amylases, Science **80**: 317, 1934.
6. Somogyi, M.: Micromethods for the Estimation of Diastase, J. Biol. Chem. **125**: 399, 1938.
7. Wohlgemuth, J.: Ueber eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments, Biochem. Ztschr. **9**: 1, 1908.
8. Somogyi, M.: Determination of Serum Amylase Activity, Proc. Soc. Exper. Biol. & Med. **32**: 538, 1934.
9. Dozzi, D. L.: Urinary Amylase—Its Estimation and Significance, Am. J. Digest. Dis. **7**: 123, 1940.
10. Small, J. C.: A Method for the Preparation of Soluble Starch, J. Am. Chem. Soc. **41**: 113, 1919.
11. Crandall, L. A., Jr.: The Origin and Significance of the Blood Serum Enzymes, Am. J. Dig. Dis. & Nutrition **2**: 230, 1935.

THE RAPID DIAGNOSIS OF MALARIA FROM THICK BLOOD SMEARS*

GUILLERMO CASTRO, D. SC., PANAMA, R. P.

IN THE tropical divisions of the United Fruit Company, malaria is the most prevalent disease. Many cases presenting the clinical symptoms of this disease appear at the hospital out-patient clinic daily, and a rapid method of blood examination is essential. A quick staining method for thick blood films, which may be completed in seven minutes or less, is described herein. Prior to the development of this method, dispensary patients suspected of having malaria were given treatment for it, and told to return the following day for further instruction after ascertaining the results of their blood examination. The old method of preparing, staining, and examining the thick films required about two hours. Under the present method, patients need wait only a few minutes for reports on their bloods.

The latest complete publication on malaria which has come to our attention is that of Nocht and Mayer,[1] published in 1937. Concerning thick smears, the authors state on page 127: "All preparations must be allowed to dry completely . . . before further treatment . . . they must not be used for half an hour." On page 129: ". . . stain the unfixed, well dried preparation for half an hour with Giemsa's stain as described . . . slight warming or the use of a fan ventilator accelerates drying."

*From the Chiriqui Land Company Hospital, Puerto Armuelles, Panama.
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From the foregoing quotation, it is obvious that more than an hour would elapse before the preparation would be ready for examination. The method recommended here is a modification of the one mentioned by Barber and Komp[2] as a "quick staining of thick films for early diagnosis." It gives us reliable results in so short a time that it has been adopted as routine in our laboratory.

PROCEDURE

1. Place a large drop of blood (about three times the amount needed for a thin film) ¼ inch from the end of a clean slide, and smear it well in a circle of about ½ inch in diameter. Place the slide on a hot plate, at about 50°-60° C., where the blood will dry in less than one minute. The top of the chalet form microscope lamp makes an excellent hot plate for this purpose. Make a semicircle with a wax pencil, enclosing the blood between the pencil mark and the edge of the slide.

2. Prepare the staining solution in the following manner: To 3 drops of old distilled water in a short tube add 1 or 2 drops of Giemsa's stain. The 4 or 5 drops of this fresh staining solution is just sufficient to cover the area enclosing the blood.

3. As soon as the blood is dry, stain for three to five minutes, with the slide in a horizontal position. Then wash very gently in a washing tray filled with tap water or, preferably, old distilled water, submerging the slide horizontally in the washing tray, moving it backwards and forwards, and removing it slowly. This will prevent the blood film from being washed away. Drain off the excess water, and stand the slide on end in an inclined and almost vertical position, on the hot plate or in front of the microscope lamp.

The slide should be removed from the hot plate the moment the smear begins to lose its moist appearance. Further heating at this point would cause decolorization. Failures are due to overheating while drying after staining. Restained smears are less satisfactory.

Our Giemsa's stain is prepared according to the directions given by Benavides:[3] "Place 2.4 gr. of Azur II eosin (Grubler) in a clean Erlenmeyer flask and then add 200 c.c. of C.P. anhydrous glycerin. The flask is covered with a well-fitted cork and then placed in a water-bath at 60 degrees C. for 30 minutes. The flask is shaken once or twice during this period. It is then removed from the bath, and 200 c.c. of C.P. acetone free methyl alcohol are added; and, after mixing the contents, the flask is again placed in the hot water-bath for another half hour—shaking it once or twice during this period. It is then removed, and left in the incubator overnight at 37 degrees C. Next day it is passed through filter paper and stored in 200 c.c. bottles that have been thoroughly cleansed, washed with methyl alcohol, and dried before use. These bottles are well stoppered and kept in a dark place."

RESULTS

The result is essentially that of the usual well-stained thick blood films. The red blood cells are completely decolorized. Schüffner's dots in large tertian parasites may be brought out in the thinner parts of the smear. Malarial pigment is readily observed. The parasites stain deeper than in the usual

thick blood films; this is especially true of the cytoplasm of tertian parasites. The leucocytes are so easily classified that we often do differential counts in our smears. The basophilic granules of some erythrocytes often seen in anemias, and especially the anemia of chronic malaria, are shown well. The presence of such basophilic staining suggests chronic malaria and calls for extensive search if parasites are not found immediately.

The time interval between the taking and the staining of the blood is of paramount importance. With an increasing interval between the taking and the staining of the blood, the stain, when used with this method, loses its ability to dehemoglobinize the red blood cells. Very poor results are obtained with bloods taken more than twelve hours in advance. Moreover, the method is not practicable for large numbers of smears (e.g., as in surveys), because each slide has to be stained separately. At the most, 4 or 5 bloods may be smeared on one slide and stained at once.

For the outpatients, and for those acutely ill and requiring prompt attention, this method is of particular value, as it permits a rapid examination and estimation of the severity of infection, the quality and approximate quantity of the leucocytes, and the response to treatment. In cases in which a prompt diagnosis is not urgent, the method of Barber and Komp may be preferred.

SUMMARY

A rapid method for the staining of thick blood films for the diagnosis of malaria, and its applicability to routine dispensary and hospital work, is described.

REFERENCES

1. Nocht, Bernard, and Mayer, Martin: Malaria, London, 1937, John Bale.
2. Barber, M. A., and Komp, W. H. W.: Method of Preparing and Examining Thick Films for the Diagnosis of Malaria, Pub. Health Rep. **44**: 2330, 1929.
3. Benavides, Joaquin: Comments and Procedure on Thick Blood Film Technic, J. LAB. & CLIN MED. **20**: 289, 1934.

THE DETERMINATION OF pH VALUES OF BIOLOGICAL FLUIDS

II. THE INFLUENCE OF TEMPERATURE, DILUTION, AND THE TYPE OF DILUENT UPON THE pH VALUES OF BACTERIOLOGIC MEDIA*

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INTRODUCTION

THE pH values of a number of biological products and bacteriologic media, obtained by colorimetric and electrometric methods at 25° C. and 30° C., have been presented in a recent paper.[1] Depending upon the particular colorimetric procedure employed, the test solutions were diluted with fixed quantities of distilled water or physiologic saline solution. The pH values of the undiluted test fluids, obtained with the glass electrode, were in agreement well within the limits ±0.3 pH unit, with the values of the diluted test fluids obtained by means of the different colorimetric methods. In the present work, the pH values of a number of buffer solutions and bacteriologic media, diluted with varying quantities of distilled water or saline solution, have been determined at temperatures between 10° C. and 38° C.

In order to determine the pH values of the bacteriologic media as accurately as possible at the stated temperatures, a series of pH determinations was first made on standard buffer mixtures. These data were then tested for their degree of conformity with thermodynamic principles and an approximate form of the Debye-Hückel limiting law. A comparison was also made with the pH values of these reference buffers obtained by MacInnes and Hitchcock and their co-workers at the various temperatures.[2-4] From these tests it was found that the MacInnes-Belcher glass electrode, in conjunction with the Young Electron Ray Meter, give accurate and reproducible results at the temperatures of measurement.

The general problem of pH measurement, as well as the specific applications of pH measurements to problems in bacteriology, has been fully described by Clark.[5, 6] Zimmermann has recently made a study of the errors involved in electrometric pH determinations of culture media.[7] Employing the quinhydrone electrode and saturated calomel electrode system, he studied the variation of pH with temperature for bouillon and agar bouillon media. He also measured the effect of aqueous dilution upon the pH values of the media.

The use of semi-fluid media for bacterial cultivation has presented a new problem in pH determination. It has been generally recognized that colorimetric methods do not always give satisfactory and accurate results.[8] To this end, the present study of bacteriologic media has been extended to include pH measure-

*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York.
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ments at various temperatures on a semi-fluid medium and on a semi-solid medium commonly employed in bacterial culture.

EXPERIMENTAL METHODS

The procedure adopted in this work consisted of the measurement of pH values of the test solutions set up in a galvanic cell with liquid junction:

Glass, Test Solution : KCl (saturated) : HgCl, Hg

The Young Electron Ray Meter was employed in conjunction with a MacInnes-Belcher durable condenser type glass electrode. The latter has been fully described in the literature.[9, 10] The Young Electron Ray Meter is a potentiometer capable of recording either in millivolts or pH units. In these experiments, the instrument was set to read directly in pH units. The Electron Ray Meter employs a cathode ray tube in conjunction with a five-element vacuum tube as null-point indicator.

Buffer solutions were prepared with Baker's "analyzed" salts. A 0.05 molar primary potassium phthalate ($KHC_8H_4O_4$) solution was used as a primary pH standard. This solution was prepared from the crystalline salt purchased from the National Bureau of Standards. The pH values of 0.05 molar primary potassium phthalate have been carefully determined at various temperatures by MacInnes and his co-workers.[4] On the basis of their work, the following pH values were assigned to the standard phthalate solution and used throughout this work:

	0.05 M $KHC_8H_4O_4$						
t° C.	10	15	20	25	30	35	38
pH	4.00	4.00	4.00	4.00	4.01	4.02	4.02

The bacteriologic media were prepared from the best ingredients available, according to the methods described mainly by Wadsworth.[11] Suitable precautions were observed in order to maintain the sterility of the test solutions. Dilutions were made with sterile distilled water or physiologic saline solution, and the solutions were stored in sterile 1 ounce bottles. Immediately before and after pH determinations, the test solutions were stored in the icebox at about 7° C.

pH measurements were carried out by enclosing the glass electrode filled with test fluid in an electrically grounded metal case, which in turn was enclosed in an incubator with thermostatic temperature control. By means of the latter, the temperature around the glass electrode assembly (glass and saturated calomel electrodes) was kept constant within ±0.5° C. during any measurement. When not in use, the glass electrode was filled with distilled water. By this practice, the residual potential within the glass membrane (asymmetry potential) was maintained at a low constant level.[9] pH measurements were made with sodium acetate and borate buffer mixtures in order to cover the region pH 4.0 to pH 9.0. Within this pH range, the MacInnes-Belcher glass electrode, in conjunction with the Young Electron Ray Meter, functioned accurately within the limits ±0.02 pH unit.

EXPERIMENTAL RESULTS

The pH values in Tables I and II represent the results of at least three concordant measurements.

The following are the compositions of the media employed in these experiments*:

(A) Pneumococcus broth: beef heart infusion, peptone (Parke-Davis) 1 per cent, disodium phosphate 0.2 per cent.

(B) Veal broth: veal infusion, peptone (Difco) 1 per cent, sodium chloride 0.5 per cent.

(C) Glucose beef heart phosphate broth: beef heart infusion, peptone (Parke-Davis) 2 per cent, glucose 0.5 per cent, disodium phosphate 0.2 per cent.

(E) Diphtheria broth: distilled water 7 liters, bacto-veal (Difco) 1 lb., proteose peptone 2 per cent, sodium chloride 0.5 per cent, sodium acetate 1 per cent.

(F) Hormone broth (semi-fluid): beef heart infusion, peptone 1 per cent, sodium chloride 0.5 per cent, gelatin 0.2 per cent.

(G) Glucose beef heart phosphate agar (semi-solid): beef heart infusion, peptone 1 per cent, glucose 0.03 per cent, disodium phosphate 0.2 per cent, agar 0.25 per cent.

Table I summarizes the results obtained with the acetate and borate buffer mixtures. The changes in the pH values for any one solution of the acetate buffer mixtures was 0.07 pH unit or less between 10° C. and 38° C. In column (1) is given the degree of dilution or the percentage dilution of the acetate buffer mixture with distilled water or physiologic saline (0.85 per cent NaCl) solution; columns (2) and (3) show the molar concentrations of acetic acid and sodium acetate, respectively, after aqueous or saline dilution; and column (4) shows the molar concentration of sodium chloride in the buffer solutions diluted with physiologic salt solution. The maximum and minimum pH values for the dilutions between 10° C. and 38° C. for the aqueous and saline buffer mixtures are shown in columns (5) and (6), and the average pH values between 10° C. and 38° C. for all dilutions of the buffer solutions made up with distilled water and with saline solution are shown in columns (7) and (8).

For the borate buffer mixtures, columns (1) to (3) have the same significance as in the first part of Table I. The pH values of the test solutions diluted with distilled water or physiologic saline solution at the different temperatures are presented under column (4).

From the data in Table I, it is evident that for the acetate and borate buffer mixtures, the pH values increased with increasing aqueous dilution at all temperatures studied. Dilution with physiologic saline solution, on the other hand, decreased the pH values or increased the hydrogen-ion activities of the buffers. For both the diluted and undiluted borate buffer mixtures, the pH values decreased with increasing temperature, but for the acetate mixtures, the pH values at the different temperatures remained constant within the limits of precision of the instrument. These results are in agreement with those obtained by Sörenson,[12] Kolthoff,[13] and others.[6]

*Thanks are due to Miss M. McGrath, of the Media Division, for kindly providing the media used in this work.

TABLE I

ACETIC ACID—SODIUM ACETATE BUFFERS

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
% DIL.	CH_3COOH	CH_3COONa	NaCl	RANGE OF pH VALUES 10° C.–38° C.		AVERAGE pH VALUES 10° C.–38° C.	
				H_2O DILUTION	0.85% NaCl DILUTION	H_2O DILUTION	0.85% NaCl DILUTION
0	0.05230	0.05000		4.62–4.68		4.65	
10	0.04707	0.04500	0.01454	4.62–4.68	4.63–4.69	4.66	4.66
25	0.03923	0.03750	0.03635	4.63–4.69	4.62–4.68	4.67	4.65
50	0.02615	0.02500	0.07271	4.63–4.70	4.59–4.65	4.67	4.62
75	0.01308	0.01250	0.10906	4.67–4.72	4.58–4.64	4.70	4.61
90	0.00523	0.00500	0.13087	4.74–4.77	4.58–4.64	4.76	4.61

BORAX BUFFER SOLUTIONS

(1)	(2)	(3)	(4)						
% DIL.	$Na_2B_4O_7$	NaCl	pH VALUES AT VARIOUS TEMPERATURES—t° C.						
			10	15	20	25	30	35	38
0	0.05000		9.30	9.26	9.21	9.18	9.09	9.06	9.05
10	0.04500		9.30	9.26	9.21	9.18	9.10	9.06	9.05
		0.01454		9.20	9.21	9.15	9.10	9.05	9.03
25	0.03750		9.29	9.26	9.20	9.18	9.11	9.06	9.05
		0.03635		9.16	9.18	9.14	9.09	9.03	9.00
50	0.02500		9.29	9.26	9.20	9.18	9.11	9.07	9.05
		0.07271		9.11	9.13	9.11	9.04	8.99	8.96
75	0.01250		9.30	9.27	9.21	9.19	9.11	9.07	9.08
		0.10906		9.07	9.08	9.08	9.01	8.96	8.92
90	0.00500			9.22	9.23	9.21	9.13	9.09	9.09
		0.13087		9.03	9.06	9.06	8.99	8.94	8.90

Table II summarizes the results obtained with the bacteriologic media. The first part of the table shows the pH data for aqueous dilutions of the media. The second part shows the results obtained with saline dilutions of the media. Column (1) gives the names and composition of the media; column (2) indicates the degree of dilution with distilled water or physiologic saline solution; column (3) shows the pH values at various temperatures of the diluted and undiluted media.

The pH data under column (3) in Table II show that the pH values increased with increasing aqueous dilution for pneumococcus broth, veal broth, and glucose beef heart phosphate broth (pH unadjusted). In the case of diphtheria broth and glucose beef heart phosphate broth (pH adjusted colorimetrically) there was a decrease with increasing aqueous dilution. The pH values of the hormone broth and glucose beef heart phosphate agar remained fairly constant (ca. ± 0.03 pH unit), with either aqueous or saline dilution to about 50 per cent dilution. The results with saline dilution were more uniform than with aqueous dilution of the media. The hydrogen-ion activities of all the media studied increased with increasing saline dilution.

Fig. 1 illustrates graphically the linear relationship between pH and temperature for the undiluted media. Empirical pH values below 10° C. and above 38° C. may be obtained by graphical extrapolation of the appropriate data for either the diluted or undiluted media.

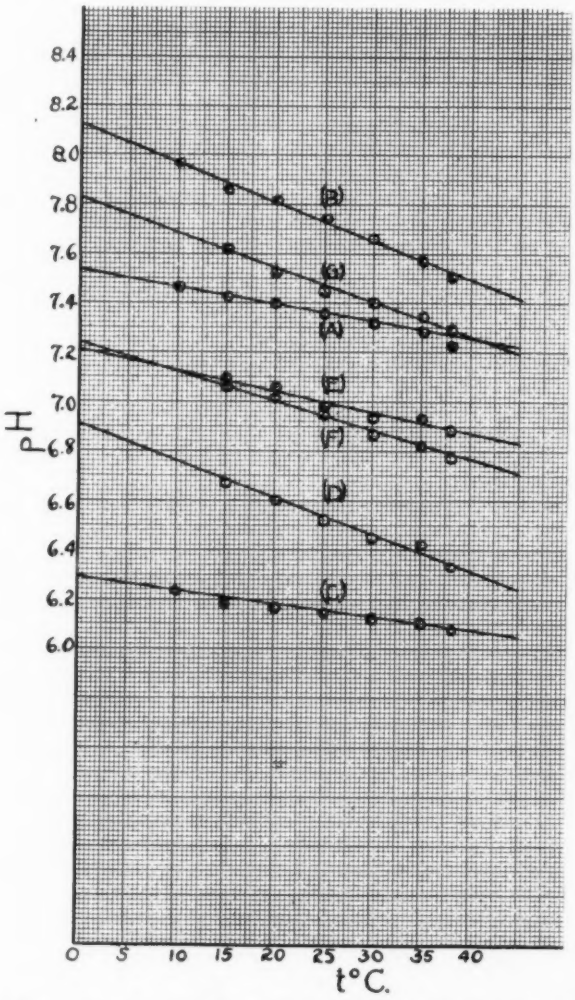

Fig. 1.—The variation of pH with temperature for the undiluted media. The letters at the end of the curves correspond to those given in Table II.

From the data at 25° C. for aqueous dilutions of glucose beef heart phosphate broth (pH unadjusted), the following linear equation has been obtained by the Method of Least Squares:

$$\frac{\text{pH}}{\text{Percentage dilution}} = 0.29 + \frac{6.14}{\text{Percentage dilution}} \qquad (1)$$

By rearranging the terms in equation (1) and substituting the appropriate values for the percentage dilution, the pH values of this medium at different degrees of dilution have been computed. The average difference between the observed and calculated pH values at 25° C. was found to be ±0.03 pH unit. All

TABLE II

WATER DILUENT

(1)	(2)	(3)						
MEDIA	% WATER DILUTION	pH VALUES OF MEDIA AT VARIOUS TEMPERATURES—t° C.						
		10	15	20	25	30	35	38
(A) Pneumococcus broth	0	7.47	7.43	7.40	7.35	7.31	7.29	7.23
	10	7.47	7.43	7.40	7.37	7.32	7.30	7.25
	25	7.48	7.44	7.42	7.38	7.33	7.31	7.26
	50	7.51	7.46	7.45	7.42	7.37	7.34	7.29
	75	7.53	7.50	7.49	7.45	7.41	7.39	7.34
	90	7.53	7.50	7.49	7.45	7.43	7.42	7.36
(B) Veal broth	0	7.97	7.87	7.82	7.75	7.67	7.58	7.51
	10	7.97	7.87	7.83	7.75	7.68	7.59	7.51
	25	7.93	7.85	7.83	7.75	7.68	7.58	7.50
	50	7.95	7.85	7.83	7.76	7.70	7.61	7.53
	75	7.92	7.84	7.86	7.78	7.72	7.62	7.54
	90	7.77	7.77	7.83	7.74	7.69	7.62	7.53
(C) Glucose beef heart phosphate broth (pH unadjusted before measurements)	0	6.24	6.19	6.17	6.15	6.13	6.11	6.08
	10	6.24	6.21	6.19	6.17	6.13	6.11	6.08
	25	6.24	6.21	6.20	6.18	6.15	6.12	6.08
	50	6.30	6.25	6.25	6.23	6.20	6.18	6.14
	75	6.37	6.34	6.33	6.32	6.29	6.25	6.22
	90	6.43	6.40	6.39	6.37	6.35	6.30	6.26
(D) Glucose beef heart phosphate broth (pH adjusted colorimetrically)	0		7.68	7.61	7.52	7.45	7.42	7.33
	10		7.67	7.60	7.51	7.45	7.42	7.33
	25		7.67	7.60	7.51	7.45	7.42	7.33
	50		7.67	7.60	7.51	7.45	7.43	7.34
	75		7.61	7.54	7.47	7.43	7.40	7.31
(E) Diphtheria broth	0		7.06	7.01	6.90	6.85	6.84	6.79
	10		7.01	7.00	6.89	6.84	6.84	6.79
	25		7.01	6.98	6.89	6.83	6.84	6.79
	50		6.98	6.98	6.87	6.82	6.82	6.78
	75		6.97	6.95	6.85	6.82	6.84	6.80
	90		6.86	6.85	6.78	6.75	6.80	6.79
(F) Hormone broth (semi-fluid)	0		7.08	7.05	6.95	6.89	6.83	6.78
	10		7.04	7.01	6.93	6.87	6.83	6.77
	25		7.04	7.02	6.93	6.87	6.85	6.78
	50		7.05	7.03	6.95	6.89	6.86	6.80
	75		7.03	7.02	6.95	6.89	6.86	6.80
	90		6.88	6.91	6.86	6.82	6.85	6.85
(G) Glucose beef heart phosphate agar (semi-solid)	0		7.62	7.53	7.45	7.40	7.35	7.29
	10		7.57	7.50	7.42	7.39	7.33	7.29
	25		7.57	7.50	7.43	7.39	7.34	7.29
	50		7.55	7.50	7.42	7.39	7.34	7.29
	75		7.43	7.38	7.31	7.31	7.25	7.25
	90		7.28	7.26	7.22	7.27	7.25	7.21

the media studied appear to obey the linear relationship between pH/percentage dilution and the reciprocal of the percentage dilution (1/percentage dilution) for aqueous and saline dilutions of the media at all temperatures of measurements. Fig. 2 shows the results obtained with aqueous dilutions of some of the media at 25° C.

DISCUSSION

An examination of the pH data in Table I and Table II indicates that the pH values of all the test solutions decreased with increasing temperature (except for the acetate buffer mixtures). Moreover, the pH values of all test solutions decreased with increasing saline dilution at all temperatures. The results obtained for aqueous dilution were not uniform for all the media. The pH values of some of the media increased while the pH values of other media de-

TABLE II—CONT'D

0.85 PER CENT SODIUM CHLORIDE DILUENT

(1)	(2)	(3)						
MEDIA	% SALINE DILUTION	pH VALUES OF MEDIA AT VARIOUS TEMPERATURES—t° C.						
		10	15	20	25	30	35	38
(A)	0	7.47	7.43	7.40	7.35	7.31	7.29	7.23
Pneumococcus broth	10	7.45	7.40	7.39	7.35	7.32	7.29	7.24
	25	7.45	7.40	7.38	7.35	7.32	7.29	7.24
	50	7.44	7.40	7.37	7.35	7.31	7.29	7.22
	75	7.41	7.35	7.34	7.33	7.29	7.25	7.20
	90	7.34	7.30	7.31	7.30	7.25	7.22	7.18
(B)	0	7.97	7.87	7.82	7.75	7.67	7.58	7.51
Veal broth	10	7.94	7.85	7.82	7.75	7.68	7.58	7.51
	25	7.94	7.85	7.82	7.75	7.68	7.58	7.50
	50	7.88	7.85	7.82	7.73	7.66	7.56	7.48
	75	7.84	7.75	7.75	7.68	7.61	7.53	7.45
	90	7.74	7.68	7.73	7.63	7.58	7.49	7.42
(C)	0	6.24	6.19	6.17	6.15	6.13	6.11	6.08
Glucose	10	6.22	6.19	6.18	6.16	6.14	6.07	6.07
beef heart	25	6.22	6.18	6.17	6.16	6.14	6.07	6.07
phosphate broth	50	6.22	6.16	6.17	6.16	6.14	6.07	6.07
(pH unadjusted be-	75	6.21	6.16	6.16	6.16	6.13	6.08	6.06
fore measurements)	90	6.20	6.14	6.13	6.13	6.13	6.08	6.06
(D)	0		7.68	7.61	7.52	7.45	7.42	7.33
Glucose	10		7.63	7.56	7.47	7.42	7.40	7.30
beef heart	25		7.63	7.56	7.47	7.42	7.39	7.30
phosphate broth	50		7.62	7.55	7.45	7.40	7.36	7.27
(pH adjusted	75		7.55	7.48	7.39	7.34	7.32	7.23
colorimetrically)								
(E)	0		7.06	7.01	6.90	6.85	6.84	6.79
Diphtheria broth	10		7.01	6.98	6.89	6.83	6.84	6.78
	25		7.00	6.97	6.88	6.83	6.83	6.78
	50		6.97	6.95	6.85	6.81	6.82	6.77
	75		6.94	6.93	6.83	6.79	6.80	6.76
	90		6.76	6.79	6.75	6.74	6.78	6.74
(F)	0		7.08	7.05	6.95	6.89	6.83	6.78
Hormone broth	10		7.03	7.00	6.91	6.85	6.82	6.77
(semi-fluid)	25		7.04	7.02	6.93	6.87	6.83	6.78
	50		6.99	6.97	6.90	6.94	6.85	6.76
	75		6.99	6.97	6.90	6.84	6.84	6.76
	90		6.85	6.85	6.79	6.72	6.67	6.57
(G)	0		7.62	7.53	7.45	7.40	7.35	7.29
Glucose	10		7.55	7.48	7.40	7.39	7.33	7.28
beef heart	25		7.55	7.48	7.41	7.38	7.33	7.28
phosphate agar	50		7.54	7.48	7.41	7.38	7.32	7.27
(semi-solid)	75		7.28	7.26	7.22	7.25	7.25	7.21
	90		7.26	7.23	7.18	7.19	7.17	7.13

creased with increasing aqueous dilution. Up to about 50 per cent dilution with distilled water or saline solution, the changes in pH values of the media were fairly small.

While nutrient media are used between 37° C. and 38° C. for the cultivation of microorganisms, the pH values of media are generally determined at room temperature between 20° C. and 25° C. Table II shows moderate but real differences in the pH values of the media between 20° C. and 38° C. which ranged up to 0.5 pH unit. This was true for the diluted and the undiluted media. These facts must, therefore, be considered in the determination of the initial optimum pH values of media for bacterial cultivation.

In view of the preceding considerations, the linear relationship developed between pH and temperature on the one hand, and pH and the degree of dilu-

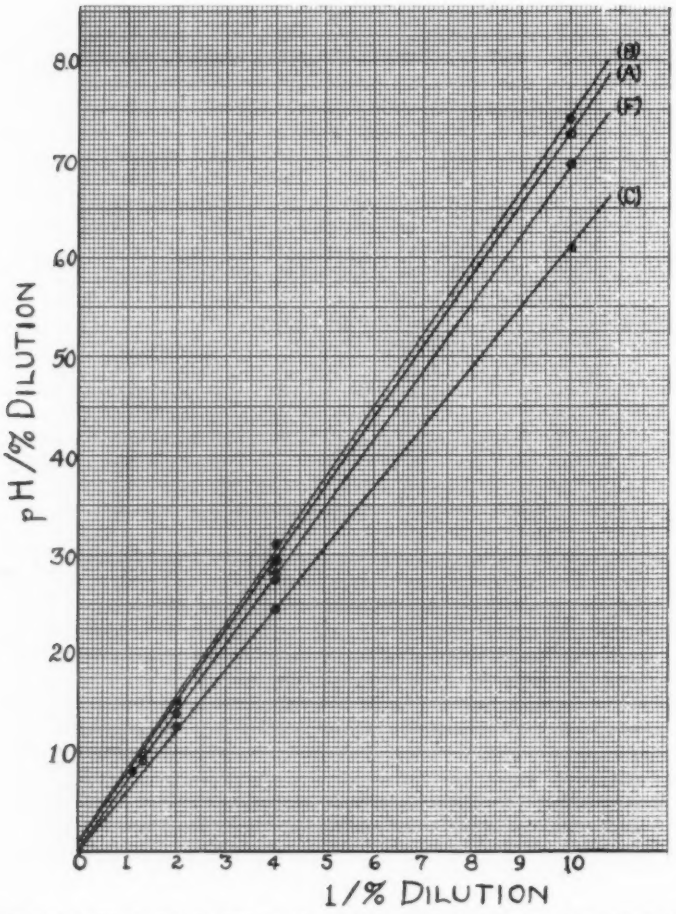

Fig. 2.—The variation of pH values of the media with the degree of aqueous dilution (per cent dilution). The letters at the end of the curves correspond to those given in Table II.

tion on the other, is useful in colorimetric determinations of the pH values of media. By the measurement of pH values of any medium at two or three temperatures, the pH values at other intermediate temperatures are predictable with a fair degree of accuracy. Although it has been shown that empirical pH values below 10° C. and above 38° C. may be obtained by extrapolation of the appropriate data, such pH values are strictly empirical and have no theoretical significance as yet. Likewise, since the dilution of test fluids is an essential procedure in the colorimetric method, the pH values of media may be determined at two or three dilutions with a specific diluent. From the relationship developed for the pH/percentage dilution and the reciprocal of the per cent dilution, pH values may be evaluated at any other dilutions with the same diluent fairly accurately.

SUMMARY

The pH values of a number of diluted and undiluted buffer solutions and bacteriologic media have been determined between 10° C. and 38° C. from measurements in a cell with liquid junction. Linear relationships for the media studied have been indicated between pH and temperature on the one hand, and

pH and degree of dilution on the other. The Young Electron Ray Meter, in conjunction with the MacInnes-Belcher durable condenser type glass electrode, has been found to yield accurate and reproducible pH values within the limits of experimental error, over the temperature and pH ranges studied.

REFERENCES

1. Glaubiger, A., and Falk, K. G.: The Determination of pH Values of Biologic Fluids. I., J. Lab. & Clin. Med. **25**: 369, 1940.
2. Hitchcock, D. I., and Taylor, A. C.: The Standardization of Hydrogen Ion Determinations. I. Hydrogen Electrode Measurements With Liquid Junction, J. Am. Chem. Soc. **59**: 1812, 1937.
3. Hitchcock, D. I., and Taylor, A. C.: The Standardization of Hydrogen Ion Determinations. II. A Standardization of the pH Scale at 38°, J. Am. Chem. Soc. **60**: 2710, 1938.
4. MacInnes, D. A., Belcher, D., and Shedlovsky, T.: The Meaning and Standardization of the pH Scale, J. Am. Chem. Soc. **60**: 1094, 1938.
5. Clark, W. M., and Lubs, H. A.: The Colorimetric Determination of Hydrogen Ion Concentration and Its Applications to Bacteriology, J. Bact. **2**: 109, 1917.
6. Clark, W. M.: The Determination of Hydrogen Ions, ed. 3, Baltimore, 1928, Williams & Wilkins Co.
7. Zimmermann, W.: Fehlerquellen bei der Einstellung und Elektrometrischen Messung der Wasserstoffionenkonzentration der Nährböden, Zentralbl. f. Bakt. **140**: 137, 1939.
8. Taylor, J. S.: A Comparison of Potentiometric and Colorimetric Methods of Determining Hydrogen Ion Concentration of Nutrient Agar, Proc. Inter. Assoc. Milk Dealers **29**: 86, 1936.
9. MacInnes, D. A., and Belcher, D.: A Durable Glass Electrode, Indust. & Engin. Chem. (Analy. Ed.) **5**: 199, 1933.
10. MacInnes, D. A.: Principles of Electrochemistry, New York, 1939, Reinhold Publishing Corp.
11. Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, Baltimore, 1927, Williams & Wilkins Co.
12. Sörenson, S. P. L., and Palitzsch, S.: Über der ''Salzfehler'' bei der Colorimetrischen Messung der Wasserstoffionenkonzentration des Meerwassers, Biochem. Ztschr. **51**: 307, 1913.
13. Kolthoff, I. M., and Bosch, W.: The Influence of Neutral Salts on Acid-Salt Equilibria. I. A Contribution to the Knowledge of the Standard Value Used for Calculation on the Activity Exponent paH of the Hydrogen Ion Concentration, Rec. Trav. Chem. **46**: 180, 1927.

A PROCEDURE FOR THE ROUTINE DETERMINATION OF VITAMIN B_1 IN URINE*

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THE Hennessey and Cerecedo[1] method for determining the vitamin B_1 content of biological material has been modified to adapt it to the routine determination of vitamin B_1 in urine. A preliminary extraction with benzyl alcohol[2] is not necessary for the small amount of urine required in the determination of vitamin B_1 by the thiochrome reaction. The permutit adsorption can be regulated so that other urinary constituents do not interfere with the adsorption and elution of the vitamin. Urine samples may be run directly through simple adsorbing columns at room temperature, and a large number of determinations may be carried out simultaneously. The thiochrome reaction is not difficult to control in potassium chloride eluates from these columns, and the recovery of thiamine added to urine samples is about as good as its recovery from standard aqueous solutions (Table I).

Adsorption columns are set up with "Folin" permutit† which has been heated for two hours on a steam bath with an equal volume of 25 per cent potassium chloride kept acid to Congo red with sulfuric acid and then washed free of excess potassium by four changes of 0.1 per cent acetic acid. A column of this permutit, 1 cm. by 7 cm., is used, and the flow through it is kept between 1 and 0.5 c.c. per minute by a fine capillary at the bottom of the adsorption tube.‡ One to 10 c.c. of filtered urine made up to a total volume of 10 c.c. and acidified with acetic acid to pH 4.5 (nitrazine paper) are run through a column, and the unadsorbed urinary substances are in great part removed by washing with 20 c.c. of distilled water and 10 c.c. of hot 0.1 per cent acetic acid. The vitamin is eluted by passing over the column 15 c.c. of boiling 25 per cent potassium chloride acidified to pH 2 (Congo red) with sulfuric acid. The washing with hot acetic acid and the elution are carried out at an elevated temperature maintained by suspending the tubes in a chamber heated by an ordinary electric heat reflector. Tubes are not allowed to run dry while being heated, and fresh permutit is used with each urine sample.

Three cubic centimeters of alkaline ferricyanide (30 c.c. of 15 per cent sodium hydroxide and 1 c.c. of 1 per cent potassium ferricyanide) and 13 c.c. of freshly distilled isobutanol are added in the order given to a 5 c.c. aliquot of cooled eluate in a small separatory funnel. A trace of the yellow ferricyanide color should persist in the aqueous phase for at least ten seconds. When an occasional eluate requires ferricyanide, a drop or two of 1 per cent potassium ferricyanide may be added directly without alteration of the thiochrome yield,

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†Permutit Co., New York City.

‡Made by E. Machlett & Sons, New York City.

TABLE I

RECOVERY OF VITAMIN B_1 FROM PERMUTIT COLUMNS

DATE	TYPE OF SOLUTION	VITAMIN CONTENT IN GAMMAS	PER CENT OF ADDED VITAMIN RECOVERED
10/6	10 c.c. of aqueous solution plus 1 drop of acetic acid	2	97
	10 c.c. of urine pH 4.5	2.3	
	10 c.c. of same urine pH 4.5 plus 2 gammas of vitamin B_1	4.35	103
10/11	5 c.c. of urine pH 4.5	1.68	
	10 c.c. of same urine pH 4.5	3.26	
	5 c.c. of same urine pH 4.5 plus 2 gammas of vitamin B_1	3.45	90
	10 c.c. of same urine pH 4.5 plus 2 gammas of vitamin B_1	5.10	90
	10 c.c. of aqueous solution plus 1 drop of acetic acid	2	87
10/27	10 c.c. of urine pH 4.5	2.57	
	10 c.c. of same urine pH 4.5	2.53	
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B_1	3.45	90
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B_1	3.46	91
	10 c.c. of aqueous solution plus 1 drop of acetic acid	1	95
10/28	10 c.c. of urine pH 4.5	0.71	
	10 c.c. of same urine pH 4.5	0.66	
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B_1	1.60	92
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B_1	1.61	93

TABLE II

CONVERSION OF THIAMINE TO THIOCHROME BY ALKALINE OXIDATION AND IMMEDIATE EXTRACTION WITH ISOBUTANOL

THIAMINE IN SAMPLES, GAMMAS	THIOCHROME FLUORESCENCE IN GALVANOMETER UNITS*
0.2	10
0.5	26
1.0	50
1.0	50†
1.5	73

*0.52 gamma of a fresh thiochrome solution which has been protected from light gives 50 galvanometer units of fluorescent light when treated with alkaline ferricyanide and extracted with isobutanol. Since the molecular weight of thiamine chloride is 335, and the molecular weight of thiochrome is 262, the values given in this table represent approximately 67 per cent conversion of thiamine to thiochrome.

†An additional 0.2 c.c. of 1 per cent potassium ferricyanide was added to this sample fifteen seconds before extraction with isobutanol was begun.

providing the thiochrome is at once shaken out into the isobutanol (Table II). The thiochrome is extracted by vigorous shaking for one and one-half minutes, the layers are separated by centrifuging for one minute, and the aqueous layer is drawn off and discarded. The isobutanol is dried over a little anhydrous sodium sulfate for a moment, and the thiochrome fluorescence is measured in a Pfaltz and Bauer photoelectric fluorometer[1] standardized against known thiamine solutions.*

*The thiochrome content of these butanol extracts can also be estimated by comparison with standard thiochrome solutions in ultraviolet light. Thiochrome for making these solutions may be obtained from Merck & Co., Rahway, N. J.

TABLE III

I. Effect on contaminating blue fluorescences in urine eluates of five-minute treatment of aliquots of these eluates with 3 c.c. of alkali in atmosphere of nitrogen.

a. Fluorescence of immediate butanol extract 20	Fluorescence of butanol extract made at end of alkali treatment 7
b. Fluorescence of butanol extract of similar aliquot treated with alkali and ferricyanide and extracted at once 44	Fluorescence of butanol extract when ferricyanide is added to eluate at end of five-minute treatment with alkali in nitrogen atmosphere 44
c. 1 gamma of thiamine in potassium chloride treated as in "b" above 53	1 gamma of thiamine in potassium chloride treated as in "b" above 51

II. Differences in the behavior of isobutanol extracts from oxidized (A) and from unoxidized (B) urine eluates. All extractions are made from solutions of comparable volume and alkalinity.

A		B	
Fluorescence in galvanometer units	After shaking with 3 c.c. of alkaline ferricyanide	Fluorescence in galvanometer units	After shaking one to two minutes with 3 c.c. of alkaline ferricyanide
44	44	20	13
6	6	11	3
Similar treatment of butanol extracts from a standard solution of thiamine.			
30	30	3	11

III. Additive fluorescences obtained by extracting oxidized urine eluate with butanol previously used to extract the thiochrome obtained from 1 gamma of thiamine.

a. Fluorescence obtained from an aliquot of urine eluate using freshly distilled butanol	b. Fluorescence obtained from 1 gamma of thiamine using freshly distilled butanol	c. Fluorescence obtained by using butanol of "b" to extract another aliquot of the same urine eluate
30	52	79*

*Theoretically, with a reagent blank of 3 one would expect to obtain in (c) a fluorescence equal to (30-3) + 52 or 79.

The conversion of thiamine to thiochrome in both standard solutions and eluates by this procedure is approximately 67 per cent of the theoretical maximum for amounts of thiamine between 0.2 and 1.5 gammas (Table II). In practice the fluorometer is so adjusted that one gamma of thiamine when converted to thiochrome gives a galvanometer deflection of 54 units when the reagent blank is equal to four units. For convenience, future adjustment of the fluorometer is made against a permanent quinine standard[1] prepared to give a fluorescence equal to that obtained by the 67 per cent conversion of one gamma of thiamine to thiochrome.

Blank determinations for the thiochrome method are conventionally obtained by treating an aliquot with sodium hydroxide and extracting the alkaline solution with isobutanol. Such blanks vary considerably with different urine eluates, but probably do not represent true blanks because in them the effect of oxidation upon contaminating fluorescent material is of necessity lacking. The blue fluorescence that appears in the blanks from permutit eluates is a lighter blue than that of thiochrome, is more sensitive than thiochrome to prolonged treatment with alkali or to brief treatment with alkaline potassium ferricyanide, and for this reason, probably does not appear in the butanol extracts of oxidized eluates. As a result, blanks on urines of low vitamin B_1 content are often

greater than the thiochrome readings. When one of these blank butanols from an unoxidized urine eluate is shaken with alkaline ferricyanide, its light blue fluorescence disappears and is replaced by a thiochrome-like fluorescence of about the same order of magnitude as that which is obtained when a butanol blank from a standard solution of similar vitamin B_1 content is shaken with 3 c.c. of alkaline ferricyanide. On the other hand, the fluorescence of butanol extracts from oxidized eluates is like the fluorescence of pure thiochrome extracts

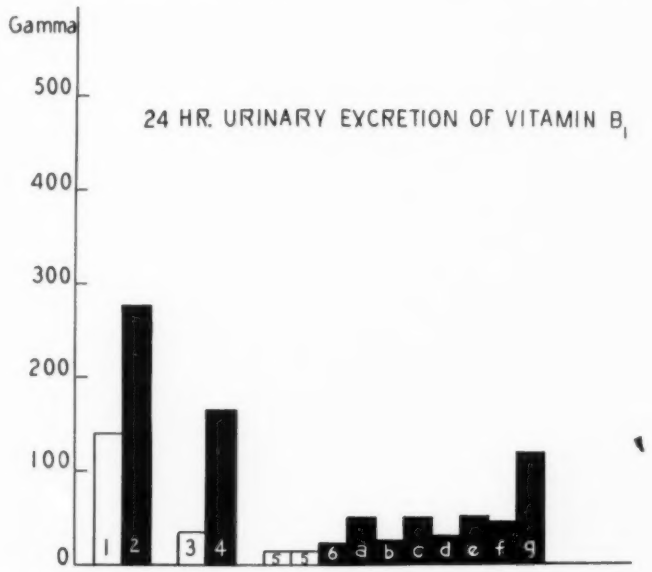

Fig. 1.—*1*, Average twenty-four-hour urinary excretion of vitamin B_1 in 10 interns. *2*, Average response of *1* to 500 γ of vitamin B_1 subcutaneously. *3*, Average twenty-four-hour urinary excretion of vitamin B_1 in 5 patients with temporarily inadequate intake as a result of recent acute illness. *4*, Average response of *3* to 500 γ of vitamin B_1 subcutaneously. *5*, Twenty-four-hour urinary excretion of vitamin B_1 in a patient with clinical vitamin B_1 deficiency. *6*, *a* - *e* response of *5* to daily injections of 500 γ of vitamin B_1 subcutaneously; *f* and *g* response of *5* to daily injections of 1,000 γ of vitamin B_1 subcutaneously.

in that it is not altered by reshaking with 3 c.c. of alkaline ferricyanide. Butanol extracts from oxidized eluates fluoresce with what appears to be a pure thiochrome color and seem to contain no materials interfering with thiochrome fluorescence. The fluorescences obtained by extracting separate portions of oxidized urine eluate with butanol and with butanol plus a known amount of thiochrome give strictly additive readings. This is contrary to the experience of Westenbrink[3] with franconite eluates. As a result of these circumstances, which are illustrated in Table III, we have felt it preferable to omit determination of the blank and to substitute an arbitrary blank deduction equal to the reagent blank.

DISCUSSION

During the past year we have made a large number of determinations of the urinary excretion of vitamin B_1 using permutit adsorption columns. Recovery of thiamine added to these urines has been 95 ±15 per cent. Recently, using the procedure as above outlined, recoveries have averaged 92 ±5 per cent. The chief source of error in the method appears to lie in the eluting process

which probably could be improved by a better control of temperature and rate of flow. Since the clinical variations which appear to be diagnostically significant are considerably greater than those inherent in the method (Fig. 1) we have not felt it necessary to complicate the present procedure by further refinements. It might be mentioned that these results are more consistent than those we obtained by omitting the permutit adsorption and substituting a more time-consuming procedure similar to that recently perfected by Wang and Harris.[4]

Our experience with the stability of vitamin B_1 in urine has been similar to that of Melnick and Fields.[2] The vitamin B_1 content of urine is not altered by twelve hours' incubation even when its pH is somewhat on the alkaline side, and it appears that the twenty-four-hour urinary excretion of vitamin B_1 is, under most circumstances, a fairly reliable index of the patient's vitamin B_1 intake. However, when this excretion is abnormally low, less than 50 gammas per twenty-four hours, it is important to distinguish between cases in which this is due to a temporarily inadequate vitamin B_1 intake, such as may occur in acute illness, and cases in which the low vitamin B_1 excretion represents a real deficiency (Fig. 1). A vitamin B_1 tolerance test appears to be one way of solving this difficulty and work of this type is now in progress.

SUMMARY

A simple procedure for determining the urinary excretion of vitamin B_1 is described.

The accuracy of the method and its application to the study of vitamin B_1 metabolism in patients are discussed.

We wish to thank Dr. D. J. Hennessey, of the Department of Chemistry of Fordham University, for help in this work.

REFERENCES

1. J. Am. Chem. Soc. **61**: 179, 1939.
2. J. Biol. Chem. **130**: 97, 1939.
3. The Determination of Aneurin (Vitamin B_1) in Urine by the Thiochrome Reaction, Recueil des Travaux Chimiques des Pays-Bas. **56**: 803, 1937.
4. Biochem. J. **33**: 1356, 1939.

THE MEASUREMENT OF CELL VOLUME OF BLOOD BY THE CELL OPACITY METHOD*

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THE problem of the volume of erythrocytes in blood is one of widespread interest. The variations under both physiologic and pathologic conditions have been widely studied for a long time. A great variety of methods have been devised for the estimation of cell volume. The data have been reviewed at length by Ponder[1] in his monograph, and by Guest[2] and Wintrobe.[3] Because of the difficulties involved in the various methods and the controversy over their accuracy, we have developed a new procedure which we have called the cell opacity method. This is based upon the principle that light is obstructed in proportion to the volume of cells present.

The transmission of light was applied to the measurement of cell volume following the suggestion of Dr. K. A. Evelyn, whose photoelectric colorimeter[4] offers a means of determining the opacity of blood cells with accuracy, ease of manipulation, speed and economy of material. Blood is collected in a micropipette and suspended in a citrate solution. It is then exposed to light passed through a filter which transmits light in the region of 660 mμ. The amount of light transmitted is measured by its effect on a photoelectric cell and recorded on a galvanometer. The obstruction of light by particles in suspension is a very complicated phenomenon.[5] Certainly some light must be reflected, refracted, and absorbed. The size and shape of the particles affect the transmission of light. It appears, however, that blood constitutes a system sufficiently constant that an empirical relationship may be established between cell volume and galvanometer readings. Although interference with the transmission of light is in itself a basic physical phenomenon of undoubted significance, this paper deals only with the correlation of the degree of opacity with cell volume.

Preliminary studies showed a direct relationship between cell opacity and the volume of blood used. In order to interpret the values obtained by the photocolorimeter, it was necessary to make measurements of cell volume on the same blood by another method. Of all the methods described, the hematocrit method, in which the relative volume of blood cells and plasma is determined by centrifugalization, is in most general use. This method was accepted for this study as the standard of reference, and an extensive series of comparisons was made.

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TECHNIQUE

The procedure adopted for the hematocrit method is as follows: Two to 3 c.c. of blood are obtained by venepuncture or heel stab and are either oxalated or heparinized to prevent coagulation. The oxalate solution contains 3 per cent ammonium oxalate and 2 per cent potassium oxalate; 0.04 c.c. per cubic centimeter of blood gives a final concentration of 0.2 per cent of oxalate.[6] The desired amount of solution is evaporated on the walls of a calibrated tube in which the blood is to be collected, so that the blood will not be diluted. If heparin is used, 1 mg. per cubic centimeter of blood is required. After mixing, the uncoagulated blood is placed in a Wintrobe tube[7] or a Rourke-Ernstene tube,[8] capped to prevent evaporation, and centrifuged at 3,000 r.p.m. for thirty minutes.

Centrifugalization causes a sharp separation of erythrocytes and leucocytes from the plasma. The volume of red blood cells alone or of the combined red and white blood cells is then read in per cent of the total volume. Inasmuch as the white cells ordinarily occupy a volume of about 0.5 per cent of the blood, for most purposes it is immaterial whether one reads the red cell volume or the total cell volume. In this study the volume of the erythrocytes alone was used.

The technique for the cell opacity method, adopted after considerable trial, is as follows: For this determination alone blood may be obtained without anticoagulants by skin puncture. If, however, a comparison is to be made with the hematocrit method, a portion of the blood containing anticoagulant, as described above, is used. Twenty cubic millimeters of blood are measured in a micropipette, calibrated "to contain." Ten cubic millimeters may be used, and the cell volume may be interpreted from the same chart if the result is multiplied by two. It is then delivered into a tube which contains 8 c.c. of citrate solution, and the pipette is rinsed with the solution.* The tubes used must be of a size suitable for the Evelyn colorimeter, and tested for uniformity. The citrate solution contains 3.0 per cent of sodium citrate (41.5 Gm. per liter of sodium citrate .5½ H_2O). Before this solution is diluted to the final volume, 1 c.c. of formalin (36 to 38 per cent formaldehyde gas) is added for each liter of solution.

A tube containing 8 c.c. of the citrate solution is put into the photoelectric colorimeter with filter "660" of Evelyn. The setting is adjusted to give a galvanometer reading of 100. The tube containing the blood in citrate solution is then rotated to give an even suspension and is substituted for the "blank" tube. The galvanometer reading is not very steady at first, and deflects toward a lower number. After fifteen to thirty seconds it becomes quite constant and does not alter for a number of minutes. The value is then translated into terms of cell volume by reading from a plot on semilogarithmic paper, an alignment chart, or a table, according to convenience (see Fig. 1 and Table I). The establishment of these standards is described below.

RESULTS

The method has a high degree of reproducibility. Because the procedure is so easy to carry out, 15 determinations were made on aliquots of the same

*The colorimeter available at present is designed for either 6 or 10 c.c. of solution. To obtain the dilution given (1/400) it is necessary to use 25 c. mm. of blood. If 20 c. mm. are used in 10 c.c. of diluent, the values given for cell volume must be multiplied by 5/4. If 10 c. mm. are used in 6 c.c. of diluent, the values given must be multiplied by 3/2.

blood. The galvanometer readings did not vary more than half a point from the average in any sample.

We have made numerous comparisons of the values obtained by the hematocrit method and the cell opacity method with both normal and abnormal bloods. In this communication we report the results of only one series of determinations.

Through the kindness of Dr. John P. Hubbard and Miss Virginia Vogel, we were able to make observations on the blood of children attending the rheumatic heart clinic of the Children's Hospital. Many of the cases were convalescent or quiescent, and none were bed patients. Each child was subjected to venepuncture without undue stasis, and the blood was delivered from an all-glass syringe directly into a tube containing dry oxalate as described above.

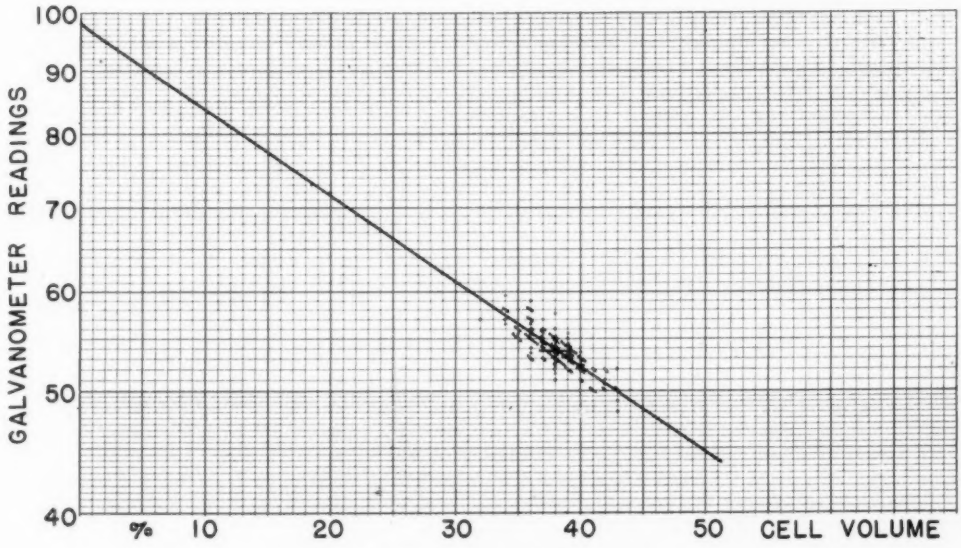

Fig. 1.—For description see text.

A series of 152 consecutive observations on blood samples examined for both cell volume and cell opacity is presented. No determinations were rejected. Refinements in technique for either method were not attempted; the hematocrit readings were recorded to the nearest half per cent of the volume of blood, and the cell opacity measurements to the nearest half mark on the galvanometer. These two values were plotted on semilogarithmic paper (see Fig. 1). The ordinates represent the logarithm of the galvanometer reading, and the abscissas represent the cell volume in per cent as determined by the hematocrit method. The point of intersection of the average cell volume and the average galvanometer reading was plotted and a line was drawn through this point to 98 on the ordinate. (With 100 per cent plasma and no cells the average colorimeter reading is 98.)

When the points showing the relationship of the cell opacity to the cell volume by the hematocrit method are plotted for each of the 152 cases, they are distributed very closely about the line in most cases, as shown in Fig. 1. Many of the points are superimposed and hence obscured, so the chart gives only an idea of the scatter.

A frequency histogram was then made to evaluate the divergence between the cell volume and cell opacity calculated as cell volume. In 51 cases the cell volumes derived from cell opacity measurement were in agreement with the hematocrit values within 0.5 volumes per 100 volumes of blood. In 32 and 34 cases, respectively, the cell opacity measurements showed a deviation of +1 and –1; in 10 and 13 cases, +2 and –2; in 4 and 3 cases, +3 and –3; in 2 and 3 cases, +4 and –4 volumes per 100 volumes of blood. No single determination gave a wider variation than this. These data when plotted (not given) show that the plus and minus errors form a close approximation to the probability curve of casual errors, and that over two-thirds of the cases are in agreement ±1 volume per 100 volumes of blood, or within 2.5 per cent of the cell volume. According to Ponder, the hematocrit method is always correct ±5 per cent, and most investigators claim a probable accuracy of 2 to 3 per cent. Inasmuch as the total variation found when the two methods are compared lies within the error of the hematocrit method alone, it is impossible to state the exact accuracy of the cell opacity method. It seems warranted to draw the conclusion that *in relatively normal cases* the accuracy of the cell opacity method approaches 1 per cent. Therefore, the values given in Fig. 1 and Table I may be considered valid as a standard for interpretation of cell opacity measurements in terms of cell volume.

TABLE I

CELL OPACITY GALVANOMETER READING	CELL VOLUME PER CENT	CELL OPACITY GALVANOMETER READING	CELL VOLUME PER CENT
85	9.5		
84	10.1	64	27.2
83	10.8	63	28.2
82	11.6	62	29.2
81	12.4	61	30.2
80	13.2	60	31.2
79	14.0	59	32.3
78	14.8	58	33.4
77	15.6	57	34.5
76	16.4	56	35.6
75	17.2	55	36.8
74	18.0	54	38.0
73	18.9	53	39.2
72	19.8	52	40.4
71	20.7	51	41.7
70	21.6	50	43.0
69	22.5	49	44.3
68	23.4	48	45.6
67	24.3	47	46.9
66	25.2	46	48.3
65	26.2	45	49.7

DISCUSSION

Hematocrit Method.—The hematocrit method which has been used as a standard for comparison with the cell opacity method is itself open to criticism. The disadvantages of this method for clinical use are that, compared to the cell opacity method, it is time-consuming and laborious and requires a considerable amount of blood. It is often inconvenient and difficult to obtain so much blood from infants. It is highly desirable that cell volume be measured without resort

to venepuncture, and on small samples such as can be obtained from a finger prick or heel stab (or from small animals). Moreover, there is no general agreement as to the correct procedure even with this simple method. Different sizes and shapes of tubes have been employed. Various anticoagulants are said to cause swelling or shrinking of the cells. Different speeds and the duration of centrifuging also give variable results. Ponder states that various bloods act differently, so that the "correct" speed and time differ. In general, it has, however, come to be accepted that when blood is centrifuged at 3,000 r.p.m. for 30 minutes, the cells occupy a volume which can be called the cell volume. This value has been considered as accurate enough for clinical purposes, except in the study of special problems.

Under normal conditions, when the values obtained by the cell opacity and the hematocrit methods do not agree, the divergence is slight and the major error probably lies in the hematocrit determination. In any event the findings of the cell opacity method do not stand or fall by comparison to the hematocrit method, but may equally well be correlated with any other method for cell volume, and the values interpreted according to the standard adopted.

Leucocytes.—The cell opacity method registers the obstruction to the passage of light of all the components of blood. It has already been shown that the plasma alone exerts a small but definite effect on the transmission of light. The effect of hemoglobin is discussed below. In this paper we have correlated the opacity with the erythrocyte content of the blood only, but obviously the leucocyte content must also be taken into account. Ordinarily there are approximately 1,000 times as many red cells as white cells in the blood. Therefore, if the passage of light depended upon the number of cells alone, the error introduced by neglecting the white cells would be only 0.1 per cent. Owing to the larger size of the white cells, their volume is normally of the order of 1 per cent of that of the red cells. Preliminary observations have shown, however, that the opacity of a white cell emulsion corresponds more nearly to that of an equal number of red cells than to an equal volume of red cells. In any event the correction due to the white cells is so small as to be within the experimental error, and if our preliminary observations are correct, without any possible significance, except in extreme cases of leucocytosis. With refinements of technique or for special purposes, a standard could be constructed with a correction factor based upon the volume or number of the leucocytes.

In the unusual or rare case in which white cells are greatly increased, as in leucemia, caution should be used in application of the cell opacity method for measurement of cell volume. Too few cases are available at present for accurate analysis of this factor. When the leucocytes are increased to approximately half a million per cubic millimeter and the erythrocytes are diminished to two to three million, the volume of the white cells may be more than one quarter that of the red cells. Under such conditions the value for cell volume, as interpreted by the chart, will be greater than that for the red cells alone, but less than that for the total cell volume—red and white cells combined.

Erythrocytes.—Inasmuch as the opacity of blood is dependent mainly upon the erythrocytes, their variations must be taken into account. The two main variants of importance for discussion are number and size.

Numerous determinations of cell opacity were made, using different concentrations of blood in the final dilution. The results, interpreted in terms of volume, are directly proportional to the amount of blood used, when the concentration is not too great. The measurement of cell opacity is most accurate for cell volumes which lie in the normal or lower ranges. When the cell volume is above 45 per cent, as for example in the blood of patients with congenital heart disease and marked cyanosis, the values obtained by the measurement of cell opacity become slightly lower than those obtained by the hematocrit method—1 to 5 per cent. Apparently a greatly increased number of cells of normal size per unit volume of diluent introduces changes in the optical properties of the fluid. The practical solution is, however, simple. When the cell volume is greater than 45 per cent, one needs only to take a smaller sample of blood, e.g., 10 c. mm., or to add an additional 8 c.c. of diluent, and multiply by two the value obtained from the chart.

We have observed a few cases of hyperchromic anemia and erythroblastosis in which the red cells are of abnormally large size, or are both large and nucleated. The cell volume, as interpreted from the cell opacity method, is lower than that obtained by the hematocrit method, 3 to 15 per cent. Whether this divergence is due primarily to the increase in cell size, the variability in cell size, or to some particular element in the blood has not yet been determined. We have not studied a sufficient number of cases to warrant discussion of the effect of variation of the shape and size of erythrocytes within a single blood (anisocytosis and poikilocytosis).

Microcytosis, conversely to macrocytosis, produces cell opacity values which are interpreted as 1 to 3 per cent too high, according to the hematocrit standard. This finding is in accordance with the studies of Drabkin and Singer[5] which show that obstruction of light is partly dependent upon particle size. We have encountered no case in which the divergence is sufficient to be clinically significant. In case of doubt the determination should be checked by the hematocrit method.

In addition, the factors of cell size and cell number are interrelated. Rat blood contains about twice as many erythrocytes as human blood, but because the cells are only half as large, the cell volume is nearly the same, and the cell opacity shows only slight divergence from that of human cells.

Hemoglobin.—Drabkin and Singer[5] showed that the transmission of light through erythrocytes is affected by the hemoglobin content. The factor for hemoglobin varies with the wave length used, as can be postulated from the absorption curve. They did not report observations beyond 630 mμ. At this point the effect of hemoglobin was of the order of 1 per cent. We selected a filter which transmits light at or near 660 mμ for our study primarily because it is not sensitive to differences in hemoglobin.* This may be proved by adding saponin and ammonia to the sample and the blank. The hemolyzed blood thus obtained gives a galvanometer reading for opacity hardly greater than for plasma alone. The hemoglobin concentration, therefore, has no effect on the determination.

*In a personal communication Dr. Drabkin states that the measurements he has made at 660 mμ show that the effect of hemoglobin is negligible at this wave length.

Diluent.—Formalin was added to the citrate solution at the suggestion of Dr. Evelyn, who recommended it as giving greater uniformity and reliability to the measurement. This has been our experience also. Apparently the formalin affects the size or shape of the cells, for when it is omitted the values for cell volume calculated from the chart are diminished about one-fourth. Presumably it makes the cells more plump, but it does not alter their ability to swell or shrink.

When hematocrit determinations are made, the anticoagulant used is of importance in either increasing or decreasing the measured cell volume. In the cell opacity method, however, the effects of the anticoagulant are so minimized by the four hundredfold dilution which the blood undergoes that they need not be considered.

The blood cells vary in volume primarily with the osmotic pressure, and secondarily, with the acid-base equilibrium of the fluid in which they are suspended. Hypertonic and alkaline solutions dehydrate the cells and cause shrinkage. Hypotonic and acid solutions cause an imbibition of water and swelling of the cells. It might, therefore, be questioned whether the solution used as a medium for suspension of the cells rigidly meets the requirements of isotonicity and correct pH. This need not be discussed, however, because the answer is not relevant to the problem. The actual osmotic pressure and acidity of the diluent used are not of prime importance because all bloods are treated alike. Whether or not the diluent is isotonic, the cells are still capable of swelling and shrinking. If a diluent of different osmotic pressure and pH were used, the results should still be proportional to the volume of red cells in the blood sample. It would be necessary only to determine the relationship under the altered conditions, between the degree of opacity and the results of an accepted method for determining cell volume.

Whatever the absolute size of the cell may be under the given conditions is unimportant. It is important, however, that it should be affected in a constant and reproducible manner. The relationship of the transmission of light and the observed hematocrit value is an empirical one; the correlation of the values, as determined by the two methods, is all that is required. This agreement has been demonstrated, as presented above.

Significance of the Method.—Other methods which deal with the blood under more or less physiologic conditions of carbon dioxide and oxygen tension attempt to measure the actual percentage volume of the cells. The cell opacity method, because of the large dilution of the blood, may be said to eliminate differences in volume due to the original condition of the cells *in vivo* with regard to oxygenation or acid-base equilibrium of the plasma. This method may be regarded as measuring the cell volume under *standardized conditions.*

In certain pathologic conditions the results of the cell opacity method may not be in agreement with those of the hematocrit method. Any difference may at first appear to be due to a defect inherent in the cell opacity method. Such is not the case. Whereas ordinarily one would be wrong and the other right, these two methods measure different physical properties of the blood, and, therefore, both may be valid. Furthermore, the difference in the results obtained

by the two methods on pathologic bloods is in itself a significant datum, and may come eventually to be correlated with a difference in the state of the cells which cannot be described in terms of either method alone.

SUMMARY AND CONCLUSIONS

A single drop of blood is sufficient to determine the transmission of light (660 mμ) through a blood suspension in citrate solution.

This measurement is directly correlated with the value for cell volume determined by the hematocrit method.

Alteration in size, shape, and structure of erythrocytes causes a demonstrable deviation from the values obtained by the hematocrit method, but for nearly normal cells the accuracy of the cell volume by the cell opacity method is greater than that of the hematocrit method.

REFERENCES

1. Ponder, E.: The Mammalian Red Cell and the Properties of Haemolytic Systems, Berlin, 1934, Borntraeger. Protoplasma Monographs, Vol. 6, Chapter II.
2. Guest, G. M., and Siler, V. E.: Centrifuge Method for the Determination of the Volume of Cells in Blood, J. Lab. & Clin. Med. **19**: 757, 1934.
3. Wintrobe, M. M.: The Erythrocyte in Man, Medicine **9**: 195, 1930.
4. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. **115**: 63, 1936.
5. Drabkin, D., and Singer, R. B.: VI. A Study of the Absorption Spectra of Nonhemolysed Erythrocytes and of Scattering of Light by Suspension of Particles With a Note on the Spectrophotometric Determination of pH Within the Erythrocyte, J. Biol. Chem. **129**: 739, 1939.
6. Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, J. Lab. & Clin. Med. **19**: 777, 1934.
7. Wintrobe, M. M.: Simple and Accurate Hematocrit, J. Lab. & Clin. Med. **15**: 287, 1929.
8. Rourke, M. D., and Ernstene, A. C.: Method for Correcting Erythrocyte Sedimentation Rate for Variations in Cell Volume Percentage of Blood, J. Clin. Investigation **8**: 545, 1930.

A SIMPLE PERFUSION PUMP*

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SINCE the advent of Lindbergh's "Mechanical Heart" many physiologists have had the desire to work with this apparatus. However, the expense connected with its construction made it, in many instances, an impossible acquirement. Therefore, a plan is presented for a perfusion pump which is inexpensive and has been found satisfactory for perfusing the organs of rats, guinea pigs, and rabbits.

The apparatus, shown on Fig. 1, is constructed entirely of pyrex glass, and all connections are ground glass. The artificial media for perfusion have been well put forth by Parker[2] and have been found very satisfactory with this perfusion pump. To sterilize this apparatus it is best to separate the individual parts and wrap them in separate cloths. After sterilization is completed, the main chamber *C* is clamped to a stand and covered over with a sterile cloth

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until ready for the insertion of the organ to be perfused. The organ is dissected out of the animal under sterile conditions and washed with Tyrode's solution. The narrow end of the perfusion tube *B* is inserted into the main blood vessel of the organ and tied there with silk thread. There is a slight enlargement about 2 mm. from the delivery end of the perfusion tube to prevent the blood vessel from slipping once it is tied. The diameter of the delivery end of the perfusion tube ranges from 0.5 mm. to 3 mm., according to the size of the blood vessel.

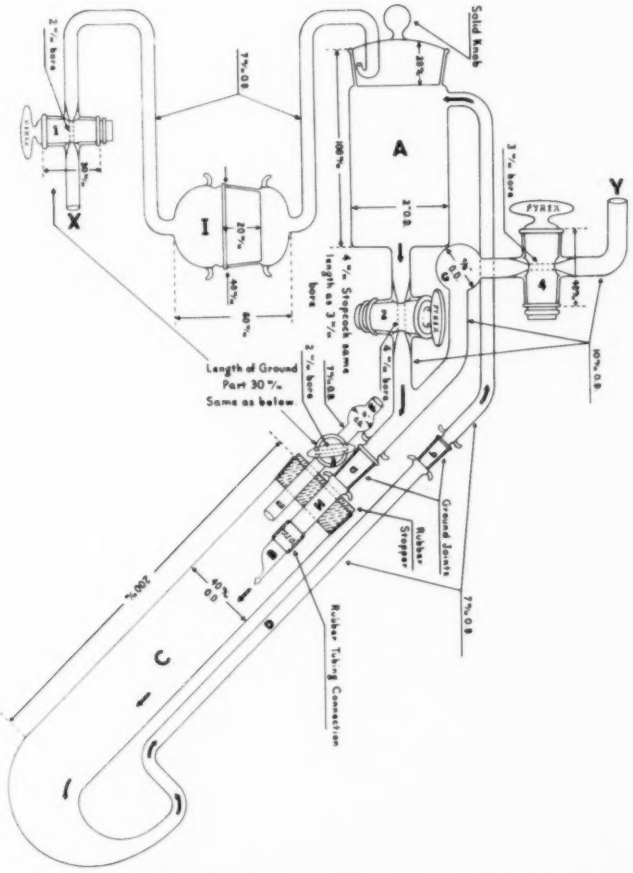

Fig. 1.—Sketch of perfusion pump showing dimensions and structural details.

A rubber bulb is filled with Tyrode's solution, attached to the wider end of the perfusion tube, and the solution is then forced through the blood vessels to clear them of blood. The rubber bulb is detached and the wider portion of the perfusion tube is inserted into the rubber tubing connection. The rubber connection is connected to the delivery tube *H* which runs through a rubber stopper. This rubber stopper is inserted into the main chamber *C*, and the reservoir *A* with its attached parts is connected at the ground glass connections *a* and *b*.

One hundred cubic centimeters of the perfusing medium is put into the reservoir *A* and then its ground glass cover is inserted in place. All stopcocks

are kept closed until operation is ready to begin. Drying chamber *I* is opened and filled with anhydrous calcium chloride and sterile cotton. The purpose of this chamber and its contents is to prevent any water vapor from coming in contact with the medium after the water suction, which is connected at *X*, has been on for a period of time. The drying chamber is closed; bulb *F* is packed loosely with sterile cotton, and the pump is ready for operation.

The entire apparatus is put into an incubator at about 37° to 39° C. and the suction at *X* is turned on. Stopcock *1* is opened halfway, allowing a slight suction to exert a pull in the reservoir *A*. Stopcock *3* is opened about halfway, so that air will enter the main chamber *C* and thus prevent the accumulation of too high a vacuum in the main chamber which would cause a back-pull on the perfusion tube. Stopcocks *2* and *4* are opened, and the air is forced out through opening *Y* which has a plug of cotton in it. The medium now flows down by gravity to the perfusing tube. After the air has been replaced by the medium, stopcock *4* is closed. Another purpose of this opening *Y* is to make possible the addition of substances without the necessity of opening the reservoir cover.

The force of gravity is sufficient to pull the fluids through the blood vessels. After the medium has passed through the organ, it flows down along the lower surface of the main chamber and as it reaches the lower portion of the bend, the suction at *X* is sufficient to draw the medium through the tube *D* and up into the reservoir chamber *A*. This circulation goes on continuously as long as there is suction at *X*.

SUMMARY

A perfusion pump is herein described that is simple in construction and has been found satisfactory for perfusing small organs.

NOTE.—Since this paper was written, a modification was made in the apparatus described here. In order to remove fluid from the pump for analysis a stopcock opening was made in the lower portion of reservoir *A*. It was also found that a small centrifugal pump inserted between ground glass joint *a* increased the perfusing efficiency of the pump.

REFERENCES

1. Lindbergh, C. A.: Apparatus for Culture of Whole Organs, J. Exper. Med. **62**: 409, 1935.
2. Parker, R. C.: Methods of Tissue Culture, New York, 1938, Paul B. Hoeber, Inc.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

PATCH TEST, A New Modification of (the Chamber Method), Rokstad, I. Arch. Dermat. & Syph. **41**: 649, 1940.

For examination of the nonspecific toxic (i.e., caustic) effect on the skin exerted by certain volatile substances (e.g., terpenes) the patch test method commonly employed in eczema is unreliable.

A new method has been worked out, therefore, in which the cellophane plate (or impregnated cambric) is replaced by a celluloid chamber which is fixed to the skin with adhesive tape (or, in cases of hypersensitiveness to the tape, with a paste made with 15 Gm. of zinc oxide, 15 Gm. of gelatin, 25 Gm. of glycerin, and 45 c.c. of distilled water).

When applied correctly, the chamber exerts a suction effect on the skin, not merely providing an airtight space that prevents escape of the volatile test substances, but inducing the formation of a papule within a certain length of time.

This papule, then indicates that the suction effect has been present; in other words, it constitutes the criterion of a successful test technique. Besides, the edematous condition of the papule may be assumed to facilitate the absorption of the test substances through the horny and sebaceous barrier of the skin.

In studies on the toxicity (i.e., the caustic effect) of the terpenes, which will be published later, this method has given constant results, revealing lower threshold values than had been previously established for erythematous or for bullous (purulent) reactions.

LUNG, Clinical Studies of Primary Carcinoma of the, Overholt, R. H., and Rumel, W. R. J. A. M. A. **114**: 735, 1940.

Primary carcinoma of the lung is a relatively common disease which is responsible for approximately 10 per cent of all cancer deaths.

In this clinical study 75 patients had histologically proved primary carcinoma of the lung.

Partial or complete bronchial occlusion, with or without secondary infection, produces the most important pathologic lesions, giving rise to early symptoms, physical manifestations, and roentgenologic changes.

Bronchoscopic examination is by far the most important diagnostic procedure available and should be used without delay in the study of any case in which a dry or productive cough develops alone or in association with hemoptysis, wheezing, dyspnea, chest discomfort, or other symptoms suggestive of early primary carcinoma of the lung that cannot be definitely explained on some other basis.

The diagnosis was established with histologic verification at an operable stage in 24 per cent of the 75 cases.

Lobectomy and pneumonectomy are practical therapeutic procedures, without an excessive operative mortality rate, which at the present time appear to offer a good chance of survival to the patient suffering from primary carcinoma of the lung.

TISSUE, Comparison of Starch Paste and Albumin Mixture as Agents for the Routine Mounting of Paraffin Sections, McDowell, A. M., and Vassos, G. A., Jr. Arch. Path. **29**: 432, 1940.

The albumin-glycerin mixture is made up by taking equal parts of egg white and glycerin, filtering, and adding a small crystal of thymol as a preservative. The starch paste tried is essentially the same as the one described by Spoerri. One gram of powdered starch is

added to 10 c.c. of cold water and thoroughly mixed; the solution is then poured into 20 c.c. of boiling water, 2 drops of dilute hydrochloric acid are added, and the suspension is boiled for five minutes while being constantly stirred to free the opalescent suspension from lumps of starch. A small crystal of thymol should be added after the paste has cooled.

The clean glass slides are prepared by coating one surface with a thin film of the mixture to be used. Drying of the affixative on the slides does not seem to be a disadvantage as long as no dust is allowed to collect on them.

Paraffin sections are then flattened out on the surface of warm water and floated onto the slides already smeared with the adhesive to be used. Sections thus mounted on slides are ready for drying prior to their deparaffinization and staining.

The table shows the method of drying adapted to varied staining techniques.

DRYING METHOD	AFFIXATIVE	MODIFIED MASSON STAIN	HEMATOXYLIN-EOSIN	MODIFIED GIEMSA	FOOT SILVER FOR BRAIN	ZIEHL-NEELSEN	FOOT SILVER FOR RETICULUM
On hot plate for 3 minutes and in drying oven at 56° to 58° C. for 1 hour	Albumin	A	A	A	A	A	I
	Starch	A	A	A	A	I	I
In drying oven at 38° to 40° C. for 24 hours	Albumin	A	A	A	A	A	I
	Starch	A	A	A	A	A	I
In drying oven at 56° to 58° C. for 48 hours or at 38° to 50° C. for 5 days	Albumin	U	U	U	U	U	A
	Starch	U	U	U	U	U	A

A = adequate; I = inadequate; U = unnecessary.

PROTHROMBIN, in the Blood of Newborn Mature and Immature Infants, Kato, K., and Poncher, H. G. J. A. M. A. **114**: 749, 1940.

The prothrombin clotting time of 173 newborn infants, both mature and immature, has been determined by the microprothrombin method; an aggregate of 1,595 tests were made during the neonatal period, extending over four weeks of postnatal life.

The average prothrombin time of 100 mature infants on the first day of life was found to be 43.2 seconds; gradually shortening as the infants grew older, by the tenth day of life the average time was twenty-five seconds.

The average prothrombin time of 73 premature infants was 46.5 seconds on the day of birth and showed a much greater degree of fluctuation on subsequent days than that of mature babies. Also in this group were some infants whose prothrombin time actually increased after birth; in one instance the prolonged prothrombin time of the blood was associated with strangulated hernia.

No correlation was found to exist between the degree of the infant's maturity and the prothrombin clotting time of the blood, at least on the day of birth.

The low prothrombin level of the blood in newborn infants satisfactorily explains the pathogenesis of hemorrhagic disease of the newborn. This may be expressed clinically as hemorrhage into the gastrointestinal tract (melena, hematemesis) or bleeding from the cord (omphalorrhagia) or genitourinary tract (hematuria). While in some cases of intracranial hemorrhage birth trauma was the precipitating factor, the severity of the bleeding was apparently induced by a lowering of the prothrombin level of the infant's blood.

GLUCOSE TOLERANCE Test, Interpretation of, Conn, J. W. Am. J. M. Sc. **199**: 555, 1940.

A short period of carbohydrate restriction in the normal person causes a marked delay in the rate of utilization of carbohydrate, as indicated by the glucose tolerance test.

Undernourished individuals respond to the same procedure with a greater loss of carbohydrate tolerance.

Erroneous diagnoses of diabetes mellitus are made when the factor of previous carbohydrate restriction is neglected.

Unless the influence of previous carbohydrate restriction is removed by means of an adequate preparatory diet, the glucose tolerance test is not reliable as an indicator of the individual's ability to utilize carbohydrate.

SULPHANILAMIDE, Significance of the Oxidation of, During Therapy, Fox, C. L., Jr. Am. J. M. Sc. **199**: 487, 1940.

The conclusion that sulfanilamide is oxidized during therapy is supported by these observations:

1. Complete, initial deprivation of oxygen interferes with bacteriostasis.

2. Increased oxygen availability magnifies bacteriostasis.

3. Reducing agents and large inocula, which rapidly reduce the potential, obliterate bacteriostasis despite excess of sulfanilamide.

4. The use of a preformed oxidant derived from sulfanilamide, 4-hydroxylamino sulfonamide, eliminates the usual initial growth period before bacteriostasis appears and gives a more prolonged effect than sulfanilamide.

5. The occurrence of methemoglobinemia during therapy and the occurrence of an oxidation product of sulfanilamide in the urine of treated patients confirm the view that an oxidant is produced in the blood or tissues of the patient.

SYPHILIS: Evaluation of the Spirochete Complement Fixation Reaction in Comparison With the Eagle Flocculation and Wassermann Procedures, Erickson, P. T., and Eagle, H. Ven. Dis. Inform. **21**: 39, 1940.

In confirmation of Gaehtgens' findings, phenolized suspensions of the Reiter strain of cultured spirochetes (palligen) react with syphilitic serum to give positive complement fixation, and the results compare favorably in both sensitivity and specificity with standard Wassermann and flocculation procedures.

In 490 sera from a group of syphilitic patients under treatment or observation, a spirochetal complement fixation technique detected 85 per cent, as compared with 68.5 per cent for an icebox Wassermann test, and 75.5 per cent for the Eagle microflocculation procedure.

Of 542 patients from a general dispensary and hospital population, with initially negative Wassermann and flocculation tests, there were 18 in whom the spirochetal complement fixation test was positive or doubtful. In no less than 14 of these could a definite diagnosis of syphilis be made on the basis of history, clinical evidence, or subsequent serologic findings.

In 88 spinal fluids the Wassermann and spirochetal complement fixation tests were both negative in 60 and both positive in 26. In 2 cases the spirochetal test only was weakly positive, and in one of these the history and clinical findings furnished strong presumptive evidence of central nervous system syphilis.

The fact that the spirochetal concentration which must be used in the test is only one-third or one-fourth the anticomplementary concentration is an obvious drawback to the routine use of the test.

BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D. J. A. M. A. **114**: 850, 1940.

Progressive hemolysis occurs in human blood stored in any of the preservatives studied.

The rate of hemolysis is much greater when blood is stored at 20° C. than at 5° C.

The addition of large quantities of isotonic dextrose solution to blood slows considerably the rate of hemolysis as compared with that in blood stored with little or no added dextrose.

The Rous-Turner blood-dextrose-citrate mixture has been so modified that it can safely be given intravenously without discarding the plasma. This modification consists of 10 volumes of blood, 13 volumes of 5.4 per cent aqueous solution of anhydrous dextrose, and 2 volumes of 3.2 per cent dihydric sodium citrate in water.

Blood stored in the modified blood-dextrose-citrate mixture will hemolyze only one-twenty-fifth to one-fiftieth as much in thirty days at 5° C. as will blood in sodium citrate alone or in sodium citrate plus sodium chloride.

The blood-dextrose-citrate mixture has proved safe and practical for human transfusions and is of distinct advantage in the operation of blood banks with a small volume of transfusions.

Hemolysis is less in blood stored in sealed flasks from which the air is completely displaced by the blood mixture than it is in blood exposed to the air.

Blood stored in flasks containing air trapped by rubber stoppers hemolyzes no faster than blood exposed to the air in cotton-plugged flasks.

Erythrocytes stored in the dextrose-citrate mixture resist destruction by shaking better than do those stored in sodium citrate alone or in citrate-saline mixtures.

The initial hemolysis encountered when blood is drawn into large volumes of preservatives can be prevented if the blood is cooled rapidly and uniformly by mixing it with preservatives which have been previously cooled to about 5° C.

BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D. J. A. M. A. **114**: 855, 1940.

There is progressive diffusion of potassium from human erythrocytes into the plasma during storage.

This diffusion is rapid during the first five days but becomes gradually less. A maximum concentration of potassium in the plasma is approached in from fifteen to twenty days.

The high values of plasma potassium attained in from fifteen to thirty days of storage cannot be accounted for by the release of that ion from completely hemolyzed cells.

Variations in the content of sodium, chloride, citrate, and dextrose in the preservative mixtures or storage at different temperatures and in various atmospheres did not affect the diffusion rate.

BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D. J. A. M. A. **114**: 858, 1940.

The high plasma potassium content in blood preserved for thirty days is not toxic when the blood mixture is transfused into human beings at velocities of less than 43.3 c.c. per minute. The concentrations of plasma potassium encountered in blood stored for one month are not high enough to cause significant increases in the serum potassium of the recipient.

BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D. J. A. M. A. **114**: 859, 1940.

A detailed study of ten patients who were given transfusions of blood mixtures varying from 25° to 15° C. and at velocities from 6.0 to 42.8 c.c. per minute revealed no significant lowering in the body temperature, no consistent changes in blood pressure, and no untoward clinical symptoms.

Preserved blood has been administered without preheating in 568 cases, with no reactions which could be attributed to the procedure.

The practice of transfusing preserved blood cold is a great convenience in conserving time and prevents untoward reactions involved in the process of heating it.

Parenteral fluids other than blood can be administered at room temperature with safety.

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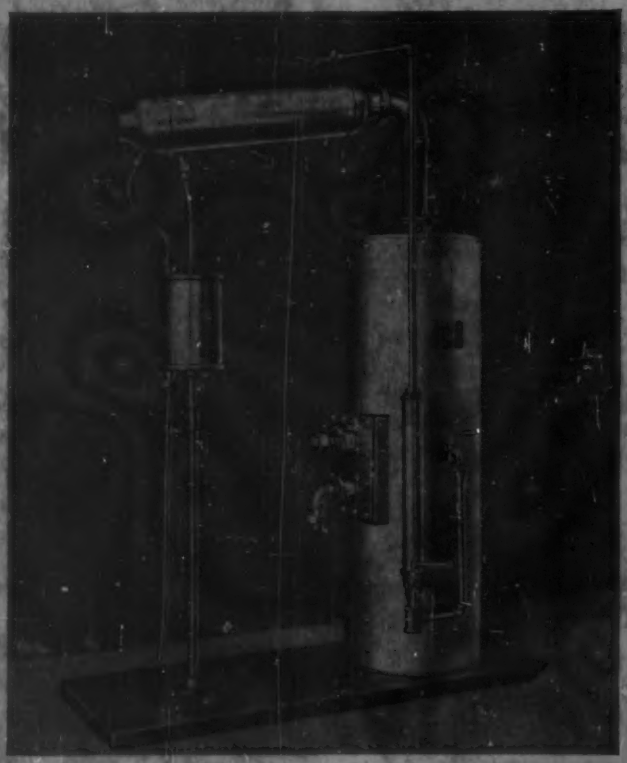

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